LUDMILA ROCHA PENONI

MORPHOLOGICAL ASPECTS OF THE *Hyalella* SMITH, 1874 (CRUSTACEA: AMPHIPODA: HYALELLIDAE) GENUS

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Tese apresentada à Universidade Federal de Lavras como parte das exigências do Programa de Pós-Graduação em Ecologia Aplicada, área de concentração em Ecologia e Conservação de Recursos Naturais em Ecossistemas Fragmentados, para obtenção do título de Doutora.

PhD. Alessandra Angélica de Pádua Bueno
Orientadora

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APROVADA em 01 de março de 2023.

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“Nós temos a responsabilidade de agir agora para minimizar nosso impacto nesse planeta – pelas nossas crianças e futuras gerações que vão herdar o que deixamos para trás.” (Paul McCartney)

RESUMO

Hyalella é o único gênero de crustáceos anfípodes encontrados nas superfícies dulcícolas de todo o território brasileiro. Quando tratamos de anfípodes dulcícolas cavernícolas, entretanto, *Hyalella* divide o espaço com outros gêneros, sendo inclusive, até o presente momento, o que parece menos adaptado para o meio subterrâneo. A morfologia dentro do gênero, principalmente considerando espécies epígeas e hipógeas encontradas no território brasileiro, é muito conservada. Ainda não há, entretanto, estudos que mostrem a influência do ambiente na morfologia de *Hyalella*. Esta tese se divide em quatro capítulos. No primeiro, encontramos que, dentre algumas variáveis ambientais, a temperatura mínima anual é a que influencia mais variáveis morfológicas, seguida pelo hábito de vida epígeo ou hipógeo. No segundo capítulo, apresentamos a descrição de uma nova espécie de *Hyalella* para a Gruta da Água Sumida e reconstruções filogenéticas de espécies brasileiras baseadas em dois genes. No terceiro, descrevemos duas novas espécies parapátricas do gênero e redescrevemos mais uma, todas as três conectadas entre si pelo rio em que ocorrem. Por fim, apresentamos mais duas novas espécies de *Hyalella* e uma chave de identificação pictórica para as espécies brasileiras subterrâneas (troglóbias ou troglófilas).

Palavras-chave: Anfípode dulcícola. Filogenia. Morfologia. Taxonomia.

ABSTRACT

Hyalella is the only genus of crustacean amphipods found on the surface waters of the Brazilian territory. When we move to the underground, however, *Hyalella* shares the space with other amphipods. It seems to be, to this day, the less adapted genus of amphipods to the underground medium. The morphology within the genus, especially amongst the Brazilian epigean and hypogean species, is extremely preserved. There isn't, to this day, any research that demonstrated the influence of the environment on the morphology of *Hyalella*. This thesis has four chapters. On the first one, we found that, among other variables, the minimum annual temperature is the environmental variable that affects more morphological variables, followed by the life habit (epigean or hypogean). On the second chapter we present the description of a new species of *Hyalella* found at “Gruta da Água Sumida” and the reconstructed phylogeny of Brazilian *Hyalella* based on two genes. On the third chapter we describe two new parapatric species of the genus and redescribe another, all of them found on the same river. Finally, we present another two new species of *Hyalella* together with a pictorial key of identification for cave related Brazilian *Hyalella* (troglobiotic and troglophile species).

Keywords: Freshwater amphipod. Morphology. Phylogeny. Taxonomy.

SUMÁRIO

PRIMEIRA PARTE

1 INTRODUÇÃO

Crustáceos são animais artrópodes que podem ser encontrados nos ambientes marinhos, dulcícolas, terrestres e semi-terrestres, tanto nos meios epígeos quanto nos hipógeos. Estes organismos possuem dois pares de antenas e o corpo é segmentado. Os representantes mais conhecidos fazem parte da classe Malacostraca, como por exemplo os caranguejos e siris, além de tatuzinhos-de-jardim e outros como os anfípodes. Há uma grande variedade de hábitos, formas, tamanhos e estilos de vida dentre os crustáceos, a saber, microcrustáceos do gênero *Daphnia* que se alimentam de plânctons e se reproduzem majoritariamente de forma assexuada, até o caranguejo-aranha-gigante, que está entre os maiores invertebrados do mundo e tem uma alimentação onívora.

Hyalella é um gênero de crustáceos anfípodes que ocorre exclusivamente no continente americano, preferencialmente em águas mais frias, mas também encontrado em regiões tropicais. De forma geral, o gênero é formado por organismos detritívoros, mas o hábito alimentar pode variar de espécie para espécie. Neste gênero completamente dulcícola encontramos animais epígeos, ou seja, de ambientes superficiais, e hipógeos, ou seja, de ambientes subterrâneos. Grande parte da riqueza está distribuída entre o sul e o sudeste do Brasil e muitas espécies são endêmicas.

A morfologia geral do gênero apresenta tanto características que são aparentemente bem conservadas entre espécies quanto características que variam bastante. Por exemplo, a relação entre o tamanho da cabeça e o tamanho do corpo, a escavação no lobo posterior da coxa 4 e a relação de tamanho entre antenas 1 e 2, sendo que a antena 2 geralmente é maior que a antena 1, parecem seguir sempre o mesmo padrão. Por outro lado, a distribuição e a quantidade de setas (estruturas cuticulares) nos apêndices, a relação de tamanho entre as pernas e entre os urópodos e formato do gnatópodos variam consideravelmente entre as espécies.

Os últimos anos foram de grande importância para o conhecimento do gênero com a descrição de diversas novas espécies. Em quase uma década a riqueza do gênero para o Brasil mais que dobrou, com novas espécies sendo reconhecidas para os estados do sul e do sudeste do país. No Brasil, o gênero pode ser encontrado nos Pampas, na Mata Atlântica, na Caatinga e no Cerrado, em nascentes, riachos e rios, dentro e fora de áreas protegidas, desde os travertinos de cavernas e até o alto de serras, abrangendo uma variedade de ambientes. As diferentes características ambientais devem refletir, de forma sutil ou consistente, na morfologia das espécies, nos hábitos alimentares e no sucesso das populações. Nas espécies de caverna, por exemplo, encontramos organismos cegos, com olhos reduzidos ou olhos aparentemente normais. Alguns apêndices tendem a se alongar também, adaptação recorrente para os mais diversos grupos de animais encontrados no meio subterrâneo.

Ao contrário de outros gêneros de anfípodes encontrados em cavernas, as populações cavernícolas de *Hyalella* no Brasil parecem suportar menores números populacionais. Não é incomum coletar menos de 10 indivíduos de *Hyalella* em uma campanha de coleta de material em cavernas em São Paulo, Minas Gerais e Paraná, por exemplo. Ao observar a distribuição do gênero pelo país e as densidades populacionais das mais diferentes espécies de *Hyalella*, esta diferença fica muito clara quando comparamos espécies epígeas e hipógeas do gênero.

Considerando a grande distribuição do gênero *Hyalella* no Brasil, a variedade de ambientes onde as suas populações são encontradas e as variações morfológicas observadas no gênero, este trabalho buscou analisar a relação entre ambiente e morfologia de *Hyalella*, além da descrição de novas espécies e a redescrição da primeira espécie troglóbia de *Hyalella* encontrada no Brasil.

Esta tese foi dividida em duas partes. Na primeira, encontramos esta introdução geral e um referencial bibliográfico. No referencial bibliográfico apresentamos uma revisão geral sobre os crustáceos e os anfípodes, detalhamento sobre a morfologia do gênero *Hyalella* e revisão sobre a influência do ambiente na morfologia de diferentes animais. A segunda parte da tese foi dividida em quatro capítulos escritos em forma de artigo: o primeiro apresenta uma análise ecológica da influência de diferentes variáveis ambientais na morfologia de *Hyalella*; no segundo artigo temos a descrição de uma nova espécie de *Hyalella* e análises moleculares com reconstrução de filogenia para espécies brasileiras do gênero; no terceiro artigo temos a descrição de duas novas espécies de *Hyalella* e a redescrição de *H. caeca* Pereira, 1989; e no último artigo apresentamos mais duas novas espécies do gênero e uma chave pictórica para identificação de espécies de *Hyalella* hipógeas ou ligadas ao ambiente subterrâneo**.**

2 REFERENCIAL TEÓRICO

2.1 Os Crustáceos

Os crustáceos compõem um dos subfilos mais diversos em todo o reino animal. Com representantes marinhos, dulcícolas, terrestres e semi terrestres, este grupo de organismos está espalhado por quase todo o planeta, exibindo uma cartela impressionante de tamanhos, formatos, cores e hábitos (Pechenik, 2016; Brusca *et al.*, 2018).

Trabalhos recentes discutem a problemática em torno do subfilo Crustacea: o grupo não é monofilético, sendo composto por três linhagens distintas. Ao unir essas três linhagens aos hexápodes, temos o táxon Pancrustacea, este, sim, monofilético (Brusca *et al.* 2018).

A classe Malacostraca abarca os organismos mais conhecidos dentro do subfilo. Aqui encontramos os caranguejos e siris, os camarões, as lagostas e os ermitões, que variam de 1 mm a mais de 50 cm (Brusca *et al.*, 2018), além de tatuzinhos-de-jardim, do *krill* e dos anfípodes, sendo estes de corpo menor, atingindo geralmente entre 7 e 12 mm de comprimento quando adultos (Rogers *et al.*, 2020).

2.2 Os Anfípodes

A ordem Amphipoda faz parte da classe Malacostraca, superordem Peracarida, e inclui cinco subordens: Gammaridea Latreille, 1802, Caprellidea Leach, 1814, Hyperiidea Milne Edwards, 1830, Ingolfiellidea Hansen, 1903 e Senticaudata Lowry & Myers, 2013 (Lowery & Myers, 2013). Estes são organismos geralmente achatados dorso-lateralmente, com corpo reduzido, sem carapaça e com um par de maxilípodos (ou seja, com o primeiro par de pernas modificado para alimentação) (Väinölä *et al.*, 2007; Brusca *et al.*, 2018).

Os Amphipoda estão distribuídos por todo o globo, ocupando principalmente os ecossistemas marinhos (Väinölä *et al.*, 2007). Cerca de 20% das espécies são dulcícolas, encontradas principalmente nos habitats mais frios e/ou subterrâneos (Väinölä *et al.*, 2007; Copilaş-Ciocianu *et al*., 2019), e dessas, grande parte compõe a subordem Senticaudata (Lowry & Myers, 2013; Rogers *et al*., 2020). Diferente de muitos crustáceos, os anfípodes não passam por um estágio larval durante o desenvolvimento, sendo que as fêmeas carregam os ovos dentro do corpo até a eclosão de juvenis (Väinölä *et al.*, 2007). Como resultado, muitas espécies dulcícolas têm uma distribuição geográfica muito pequena, podendo ser, inclusive, endêmicas (Barnard & Barnard, 1982).

Nos Senticaudata se encontram seis infraordens: Carangoliopsida Bousfield, 1977, Talitrida Rafinesque, 1815, Hadziida S. Karaman, 1943, Corophiida Leach, 1814, Bogidiellida Hertzog, 1936e Gammarida Latreille, 1802 (Lowry & Myers, 2013).

2.3 O gênero *Hyalella*

2.3.1 Conhecimentos gerais e morfologia do gênero

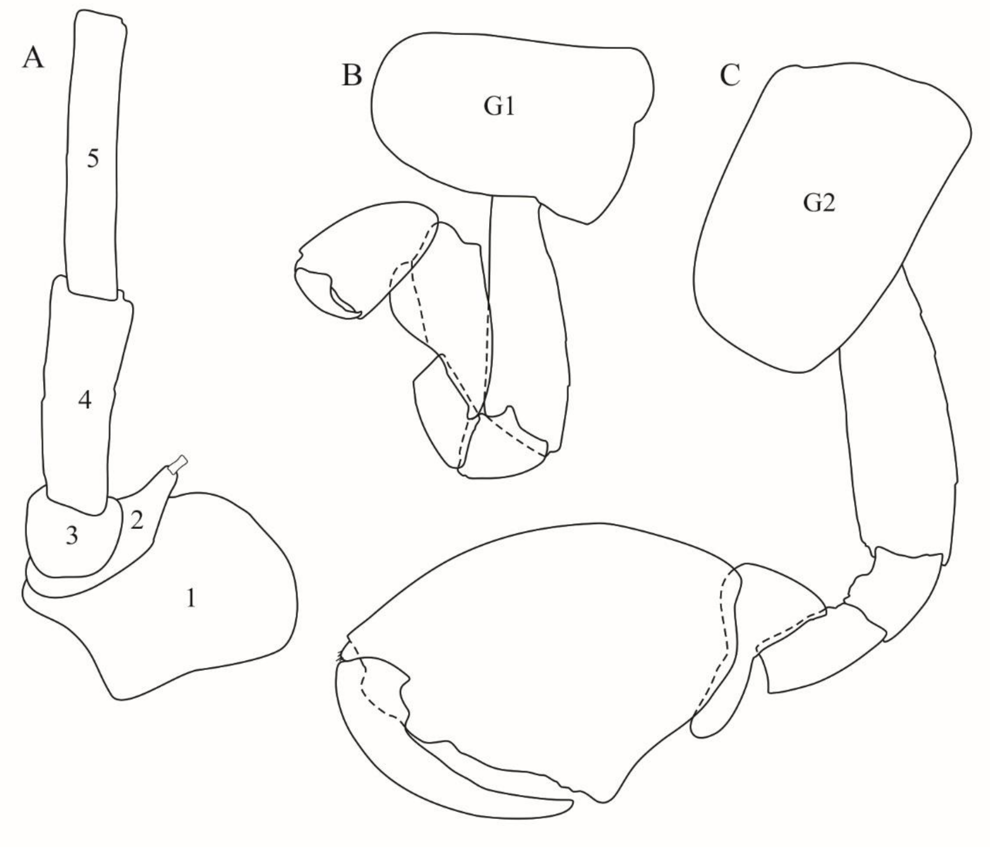
O gênero *Hyalella* é o único da família Hyalellidae Bulyčheva, 1957, que se encontra alocada na infraordem Talitrida (Rogers *et al.*, 2020). Exclusivamente dulcícola e americano, as populações do gênero são frequentemente encontradas nas nascentes, áreas alagadas, riachos, rios de 1ª a 3ª ordem e em cavernas (Grosso & Peralta, 1999; Bueno *et al.*, 2014). No Brasil, *Hyalella* é o único gênero de anfípodes encontrado nos ecossistemas dulcícolas superficiais; já nas cavernas, outras famílias de anfípodes também já foram registradas no país (Gallão & Bichuette, 2018).

A última década foi extremamente importante para o conhecimento sobre o gênero, com um acréscimo de 36 novas espécies, passando de 59 espécies conhecidas para 95 no total (Limberger *et al.*, 2022; Marróm-Becerra & Hermoso-Salazar, 2022). Dessas, 39 são brasileiras (com 21 descritas na última década), e estão distribuídas nos estados das regiões Sul e Sudeste (Penoni *et al.*, 2021; Limberger *et al.*, 2022).

Morfologicamente, *Hyalella* é caracterizado por um primeiro artículo do pedúnculo da antena 2 alargado e bulboso (Fig. 1A), pereiópodos 3 e 4 e urópodo 3 sem dimorfismo sexual, presença de brânquia coxal no 7º pereonito, e oostegitos com setas de ponta curvada (Lowry & Myers, 2013). As espécies, em geral, apresentam grandes semelhanças, e por isso, é importante usar uma combinação de características morfológicas para identificar as espécies (Bueno *et al.*, 2014). Além disso, os dois primeiros pares de pernas são modificados em gnatópodos (Fig. 1B–C) (Lowry & Myers, 2013), e o segundo gnatópodo dos machos (Fig. 1C) é bem mais desenvolvido que o da fêmea, o que torna fácil a identificação dos sexos.

Por conta da grande semelhança entre as espécies, algumas características específicas podem ser usadas para facilitar a identificação dentro do gênero. Em geral, as espécies podem ser divididas em dois grupos baseado na presença/ausência de uma seta curva no ramo interno do urópodo 1 do macho (Fig. 2A); ou então através da presença (e posição)/ausência de flanges (Fig. 2B–C), um tipo de “espinho” dorso-posterior nas placas torácicas e abdominais. Depois da observação dessas características, outras estruturas são utilizadas para diferenciar e identificar espécies em *Hyalella*.

Figura 1: Modelos de apêndices de *Hyalella* Smith, 1874. Pedúnculo da antena 2 de *Hyalella* (**A -** Artículo 1 (**1**); artículo 2 (**2**); artículo 3 (**3**); artículo 4 (**4**); artículo 5 (**5**)); Gnatópodo 1 do macho (**B**); Gnatópodo 2 do macho (**C**).



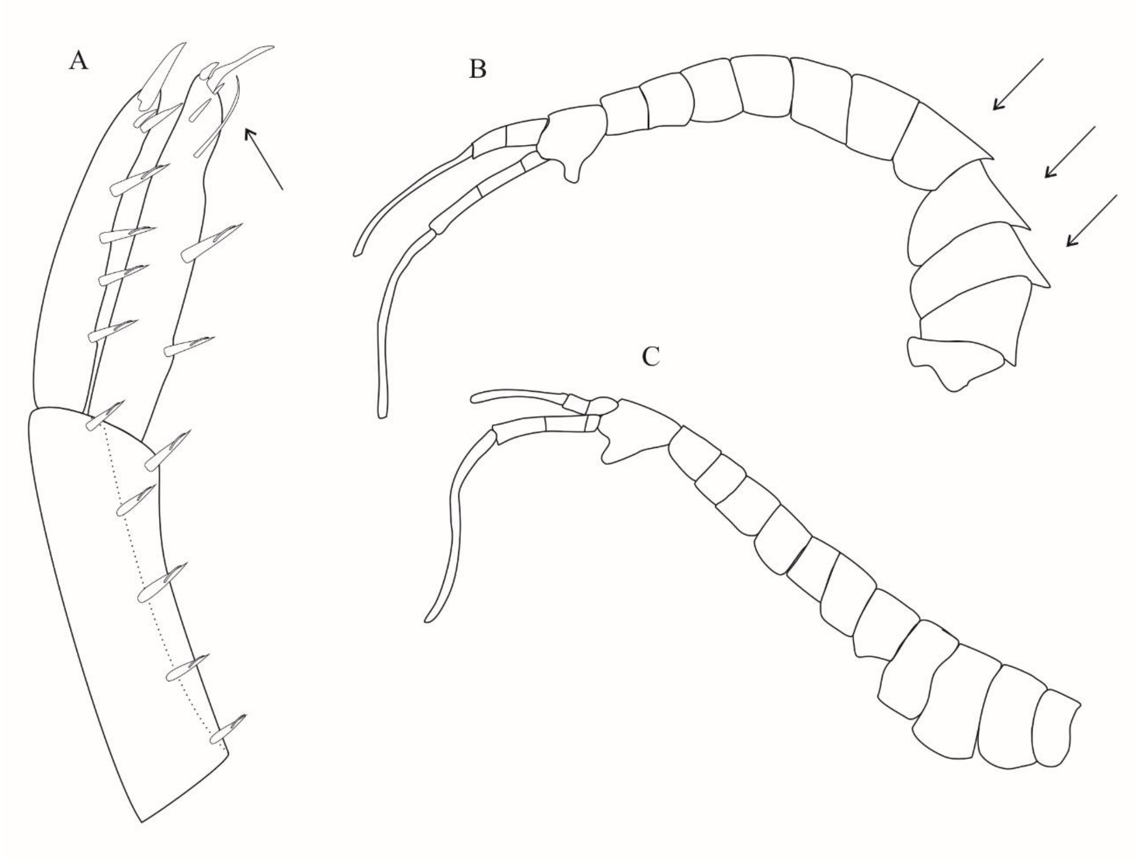
Fonte: Da autora (2023).

A seguir, revisamos todas as estruturas corporais de *Hyalella* usadas na identificação e descrição de novas espécies.

Os apêndices da cabeça são antênula (ou antena 1), antena (ou antena 2), labro (ou lábio superior), lábio (ou lábio inferior), mandíbulas direita e esquerda, maxílula (ou maxila 1), maxila (ou maxila 2) e maxilípodo.

Antena 1 (ou antênula) (Fig. 3A): dividida em pedúnculo (tri-articulado) e flagelo (n-articulado). No pedúnculo se observa a distribuição das setas e a razão dos comprimentos dos três artículos; no flagelo se observa o número de artículos – o que pode variar entre antena 1 direita e esquerda e entre os indivíduos de uma mesma espécie, e por isso se indica a variação, e a presença de estetascos (Fig. 3B), uma seta sensorial com ápice geralmente arredondado, mas largo que a base da seta e presente exclusivamente na antena 1; é importante avaliar e indicar em quais artículos os estetascos estão presentes e o número médio de estetasco por artículo. É comum medir o comprimento do pedúnculo, do flagelo, o comprimento total da antena 1, e a relação entre essas medidas umas com as outras, com o comprimento da antena 2 e do comprimento do corpo.

Figura 2: Modelos de apêndices de *Hyalella* Smith, 1874. Urópodo 1 do macho com indicação da "seta curva" (**A**); Modelo de placas torácicas e abdominais com indicação de flanges (**B**); Modelo de placas torácicas e abdominais sem flanges (**C**). Modelos **B** e **C** adaptados de González *et al.*, 2006.

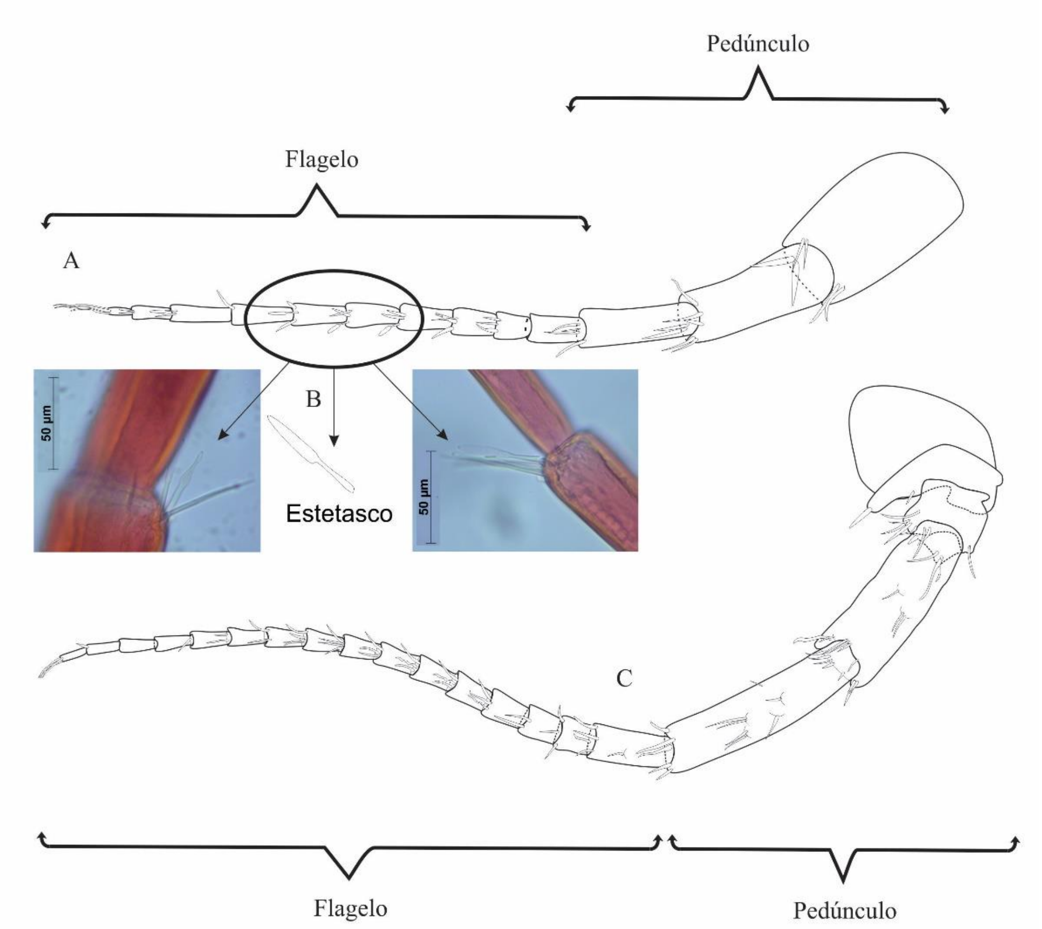


Fonte: Da autora (2023).

Antena 2 (Fig. 3C): dividida em pedúnculo (penta-articulado) e flagelo (n-articulado). No pedúnculo se observa a distribuição das setas e a razão dos comprimentos entre os artículos 3 a 5; é interessante observar com mais atenção as setas, principalmente as menores, presentes nos artículos 4 e 5; no flagelo se observa o número de artículos – o que pode variar entre antenas 2 direita e esquerda e entre os indivíduos de uma mesma espécie, e por isso se indica a variação.

Labro (ou lábio superior) (Fig. 4A) e lábio (ou lábio inferior) (Fig. 4B): essas estruturas morfológicas são muito bem preservadas dentro do gênero, tanto em formato quanto em setação. O lábio superior tem duas partes redondas separadas, com sétulas por toda a margem apical. O lábio inferior tem projeções (ou escavações) onde as mandíbulas se conectam; tem um formato de “3” ou “m”, com sétulas por toda a margem apical.

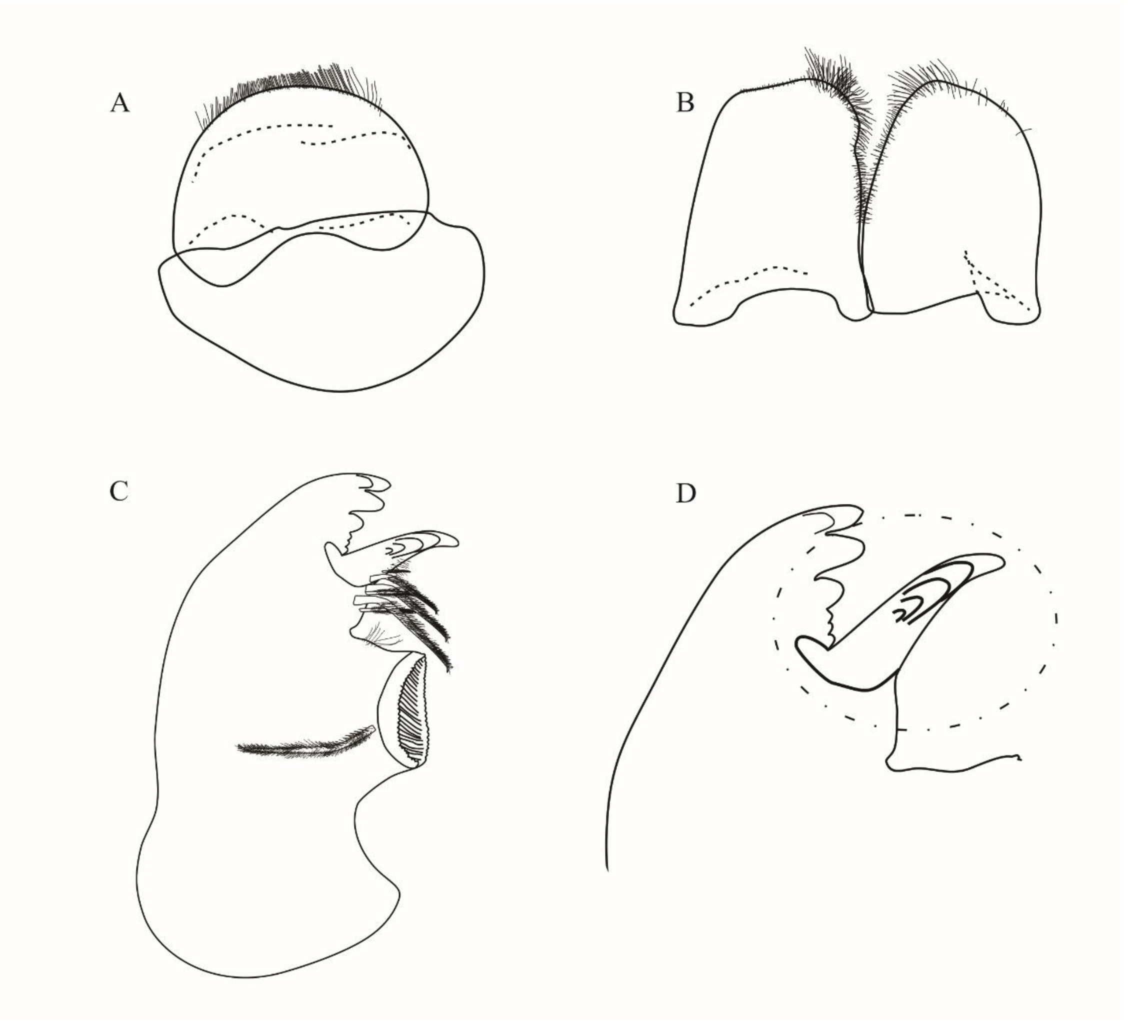
Figura 3: Modelos de apêndices de *Hyalella* Smith, 1874. Modelo de antena 1 (ou antênula), com destaque para o pedúnculo e o flagelo (**A**); Modelo e fotos de estetascos (**B**); Modelo de antena 2, com destaque para o pedúnculo e o flagelo (**C**).



Fonte: Da autora (2023).

Mandíbulas direita e esquerda (Fig. 4C): não são simétricas e apresentam diferenças importantes uma em relação à outra. A mandíbula esquerda tem uma *lacinia mobilis* (Fig. 4D) com dentes, e é importante indicar o número de dentes; também se observa o número de setas paposas ou paposserrada na fileira de setas e a presença/ausência de seta acessória do molar triturador; além disso, pode-se indicar o número de dentes no incisivo. A mandíbula direita não tem *lacinia mobilis*, mas se observa todas as outras estruturas como na mandíbula esquerda.

Figura 4: Modelos de apêndices de *Hyalella* Smith, 1874. Modelo de labro (ou lábio superior) (**A**); Modelo de lábio inferior (**B**); Modelo de mandíbulas (**C**); Destaque da *lacinia mobilis*, presente exclusivamente na mandíbula esquerda (**D**).



Fonte: Da autora (2023).

Maxílula (ou maxila 1) (Fig. 5A): dividida em ramos interno e externo. Ramo interno é mais delgado e usualmente mais curto que o ramo externo; é importante observar as setas apicais e a presença/distribuição de sétulas. Ramo externo é sempre decorado com setas serradas (Fig. 5B) – setas parecidas com pentes – e o número de setas serradas apicais é consistente entre os indivíduos na mesma espécie; aqui também se observa o palpo, seu padrão de setação e seu comprimento em relação à distância entre a base do palpo e a base das setas serradas.

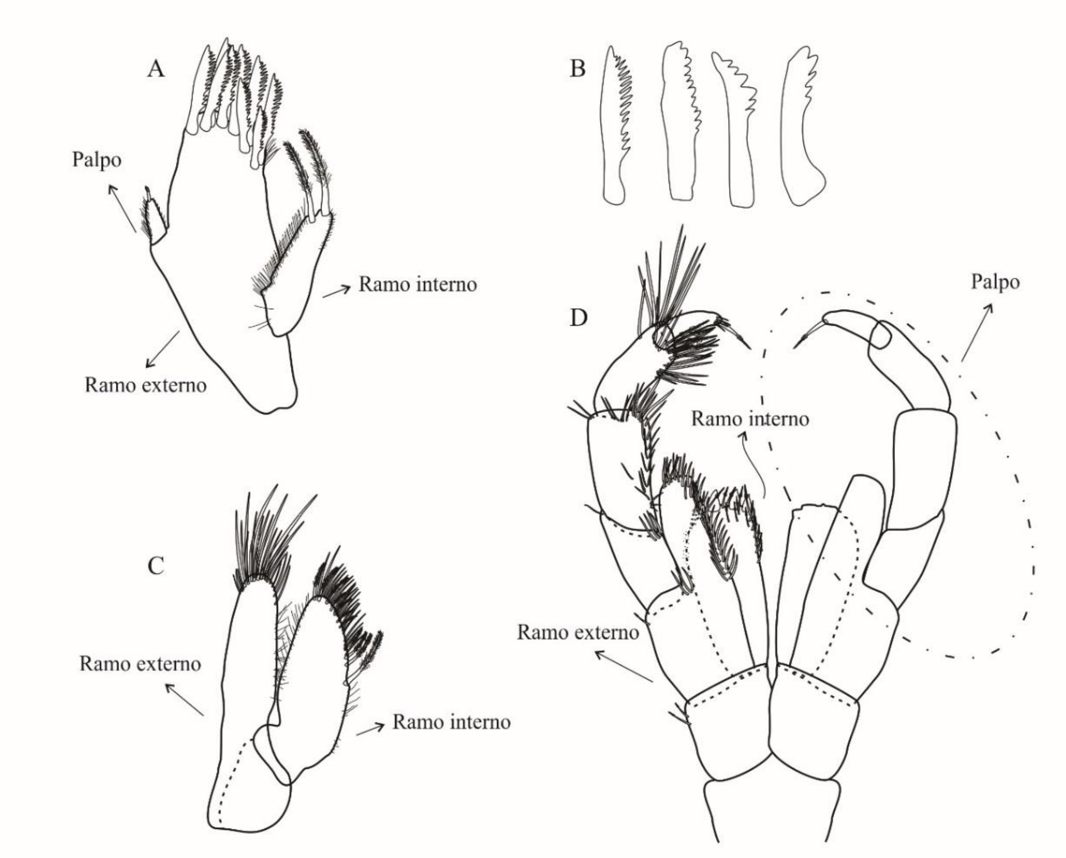
Maxila (ou maxila 2) (Fig. 5C): também dividida em ramos interno e externo. Em geral é uma estrutura cheia de setas, e muitas vezes é complicado contar o número de setas; é interessante observar o comprimento e a largura relativa entre os ramos, e descrever o melhor possível as setas observadas.

Maxilípodo (Fig. 5D): é o primeiro pereiópodo, mas que foi modificado e desempenha funções de alimentação, com os apêndices da direita e da esquerda fundidos em uma única estrutura. Direita e esquerda são simétricas, então usualmente só se desenha/descreve um dos lados. Dividida em ramos interno e externo e palpo. No ramo interno foca-se nas setas cuspidadas apicais, cônicas, e nas setas e sétulas ao redor. O ramo externo tem muitas setas principalmente na margem interna, e às vezes não é possível contar quantas são, mas se observa o tipo e o comprimento destas setas. O palpo se apoia na parte externa do ramo interno e apresenta 4 artículos; aqui se observa as setas e o comprimento relativo dos artículos; o primeiro artículo geralmente tem poucas setas na margem distal externa, e o ápice é muito mais fino que a base; os segundo e terceiro artículos são retangulares/quadrados, com variação nos padrões de setação; o quarto artículo – chamado de dáctilo – é triangular e geralmente apresenta uma seta terminal serrada ou paposa chamada de "unha".

Os apêndices do tórax (pereion = tórax) são os gnatópodos e pereiópodos. Eles estão presentes em pares (direita e esquerda) e cada um deles é dividido em sete partes: coxa, base, ísquio, mero, carpo, própodo e dáctilo. Gnatópodos 1 e 2 são pereiópodos que se modificaram em estruturas sub-queladas. Existe um importante dimorfismo sexual aqui: nos machos, o própodo do gnatópodo 2 é muito maior do que o própodo do gnatópodo 1, enquanto na fêmea os dois são quase do mesmo tamanho.

Gnatópodos 1 (Fig. 6A) e 2 (Fig. 6B–C): são observadas as setas (tipo, distribuição e número), e a presença/ausência/posição de *comb-scales* (Fig. 6D) e padrão poligonal (Fig. 6E). Algumas espécies apresentam *comb-scales* na margem posterior do mero tanto no G1 quanto no G2, e em diferentes partes dos própodos. O carpo do G2 forma uma estrutura semelhante a uma colher, com o lobo carpal afilado e projetado entre o própodo e o mero. No lobo carpal se observa a setação e a presença/ausência de *comb-scales* e padrão poligonal. No própodo do G1 e do G2 se observam os comprimentos e larguras relativos do própodo, e a relação entre os comprimentos da palma e da margem posterior. É importante observar as setas (número, tipo e posição) na face interna do própodo. No própodo do G2 pode haver uma escavação na palma, posteriormente; é importante indicar isso, detalhando se ela é delgada, profunda ou inexistente.

Figura 5: Modelos de apêndices de *Hyalella* Smith, 1874. Modelo de maxílula (ou maxila 1) (**A**); Modelos de setas serradas presentes no ramo externo da maxílula (**B**); Modelo de maxila (ou maxila 2) (**C**); Modelo de maxilípodo (**D**).



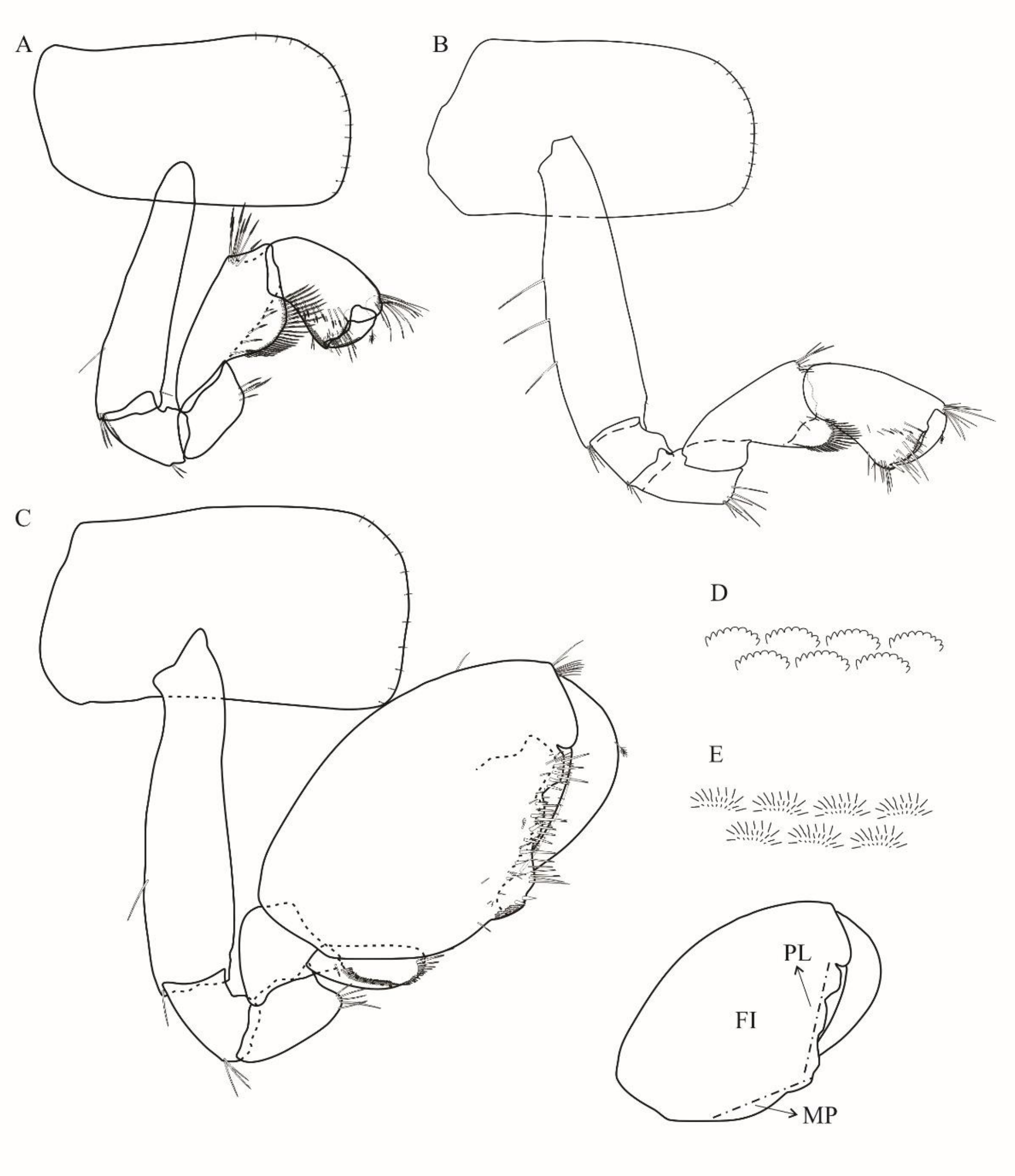
Fonte: Da autora (2023).

Pereiópodos 3 – 7 (Fig. 7A–E): são observadas as morfologias das coxas (setação e razão comprimento *x* largura), distribuição de setas, número e tipo em todos os artículos. É importante informar o comprimento relativo dos pereiópodos, indicando qual/ais é/são o(s) mais compridos.

Os apêndices abdominais (pleon = abdômen) são os pleópodos, os urópodos e o télson. São três pares de pleópodos, morfologicamente iguais, e três pares de urópodos.

Pleópodos (Fig. 8A): como os três pares são iguais, desenha-se/descreve-se apenas um deles. No pedúnculo se observa a presença/ausência de setas acopladoras (tipo ganchos) e o comprimento em relação aos ramos. Os ramos interno e externo são n-articulados e adornados com longas setas plumosas. Aqui pode-se indicar a variação do número de artículos em cada um dos ramos.

Figura 6: Modelos de apêndices de *Hyalella* Smith, 1874. Modelo de gnatópodo 1 masculino/feminino (**A**); Modelo de gnatópodo 2 feminino (**B**); Modelo de gnatópodo 2 masculino (**C**); Modelo de representação gráfica de *comb-scales* (**D**); Modelo de representação gráfica de padrão poligonal (**E**). FI = face interna; PL = palma; MP = margem posterior.

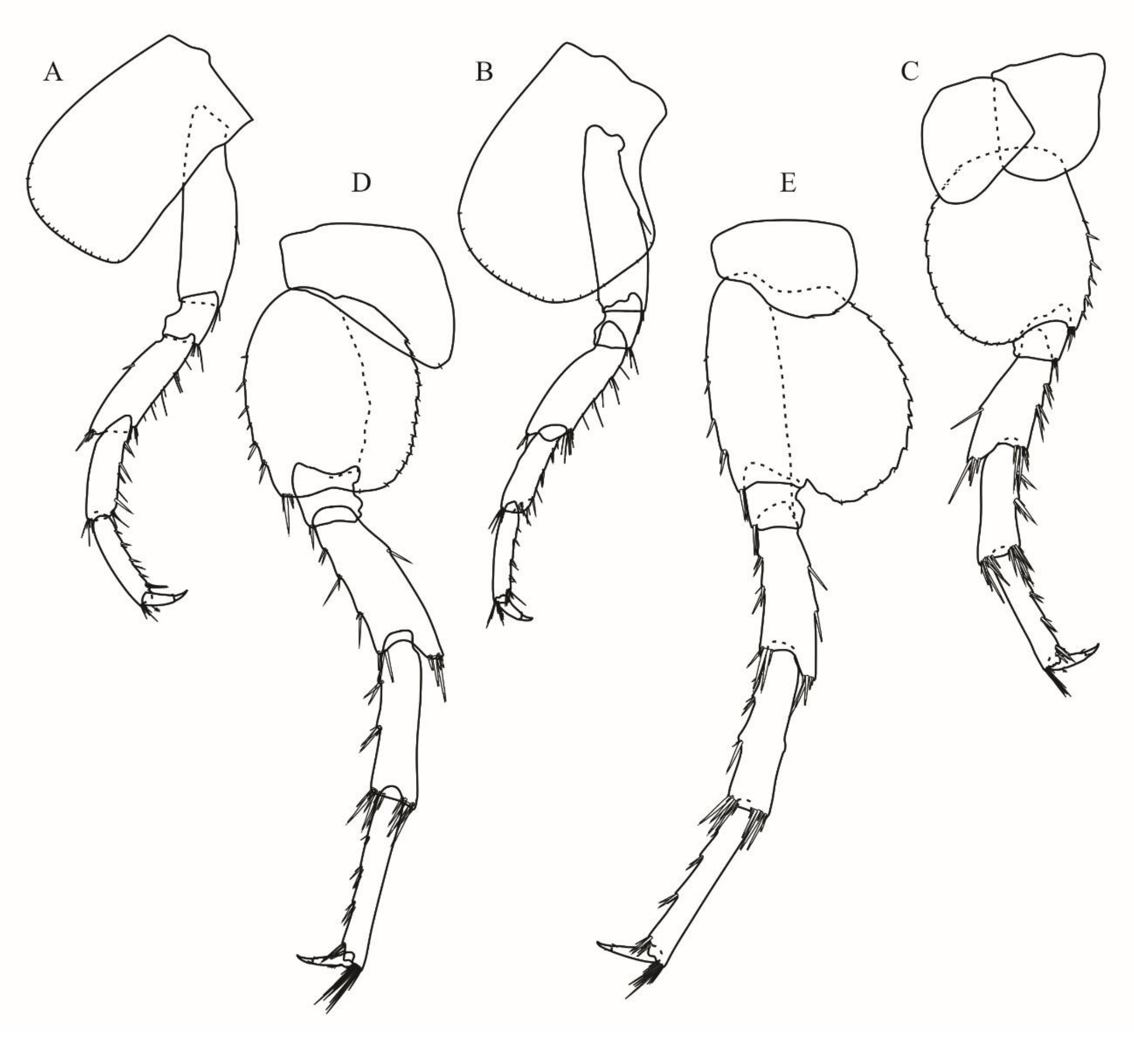


Fonte: Da autora (2023).

Urópodo 1 (Fig. 8B): estrutura birreme usualmente com diversas setas cuspidadas no pedúnculo e nos ramos. Observa-se o comprimento relativo dos ramos, o número e tipo de setas distais e apicais. Algumas espécies são caracterizadas pela presença de uma seta curva no ápice do ramo interno, apenas nos machos, o que é frequentemente utilizado para separar espécies em diferentes grupos. No caso de espécies que apresentam seta curva, pode-se observar o comprimento relativo desta seta e quaisquer ornamentações que ela possua.

Urópodo 2 (Fig. 8C): morfologicamente semelhante ao U1, mas menor. Observa-se o comprimento relativo do U2 em relação ao U1, o número, tipo e posição de setas, e comprimento relativo das setas.

Figura 7: Modelos de apêndices de *Hyalella* Smith, 1874. Modelo de pereiópodo 3 (**A**); Modelo de pereiópodo 4 (**B**); Modelo de pereiópodo 5 (**C**); Modelo de pereiópodo 6 (**D**); Modelo de pereiópodo 7 (**E**).

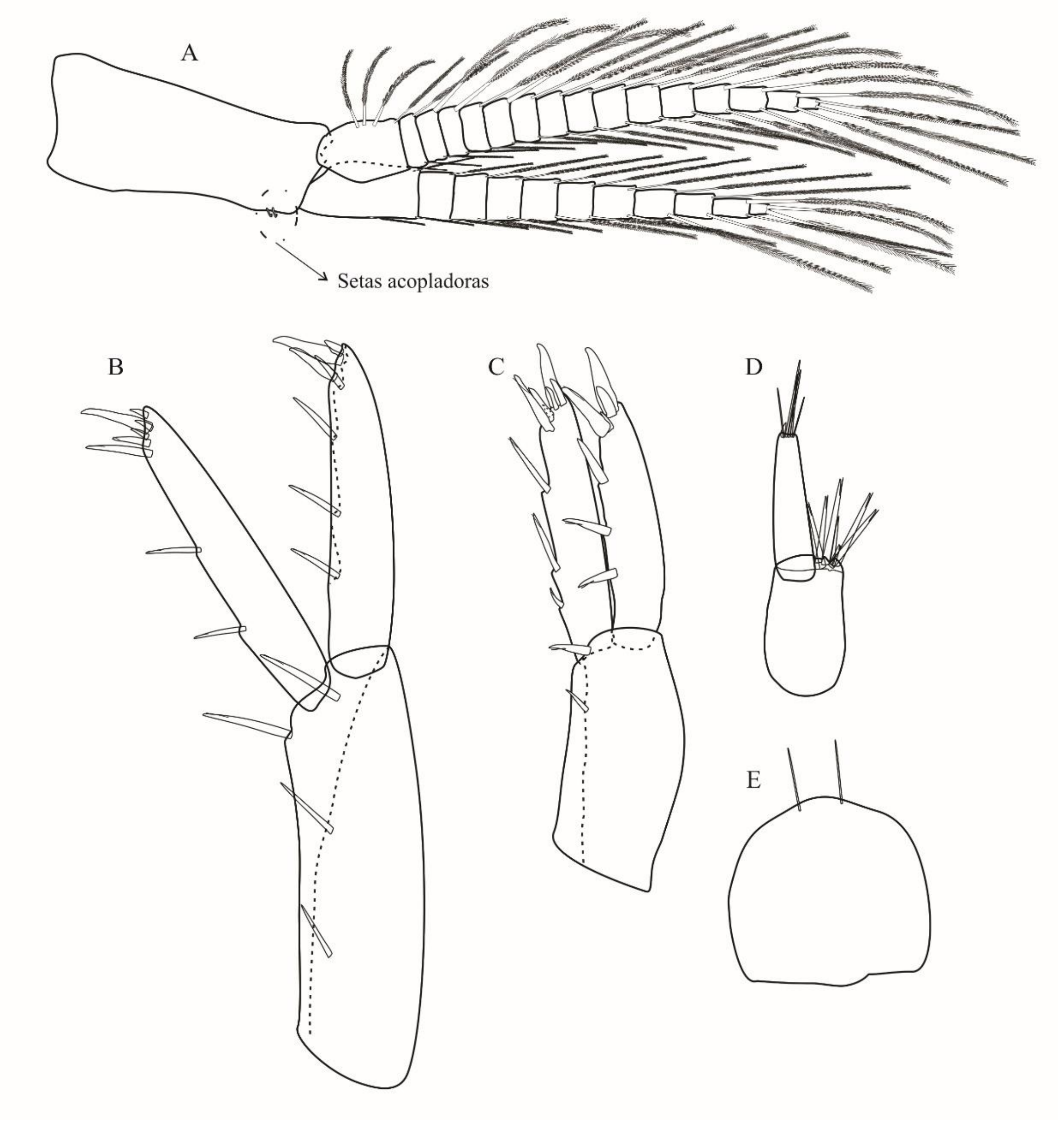


Fonte: Da autora (2023).

Urópodo 3 (Fig. 8D): unirreme (ramo interno ausente). Observa-se o comprimento relativo do ramo externo em relação ao pedúnculo, o comprimento relativo do U3 em relação ao U1, ao U2 e ao télson, e o número, tipo e posição das setas.

Télson (Fig. 8E): diferentemente de outros anfípodes, o télson de *Hyalella* é inteiro, sem divisões apicais. Observa-se a forma geral do télson e a distribuição de setas. Alguns autores indicam que a morfologia geral do télson (tamanho, forma e setas) varia bastante entre os indivíduos de uma mesma espécie – entre sexos e entre fases de vida – e por isso é importante descrever toda a variação observada.

Figura 8: Modelos de apêndices de *Hyalella* Smith, 1874. Modelo de pleópodo (**A**); Modelo de urópodo 1 (**B**); Modelo de urópodo 2 (**C**); Modelo de urópodo 3 (**D**); Modelo de télson (**E**).

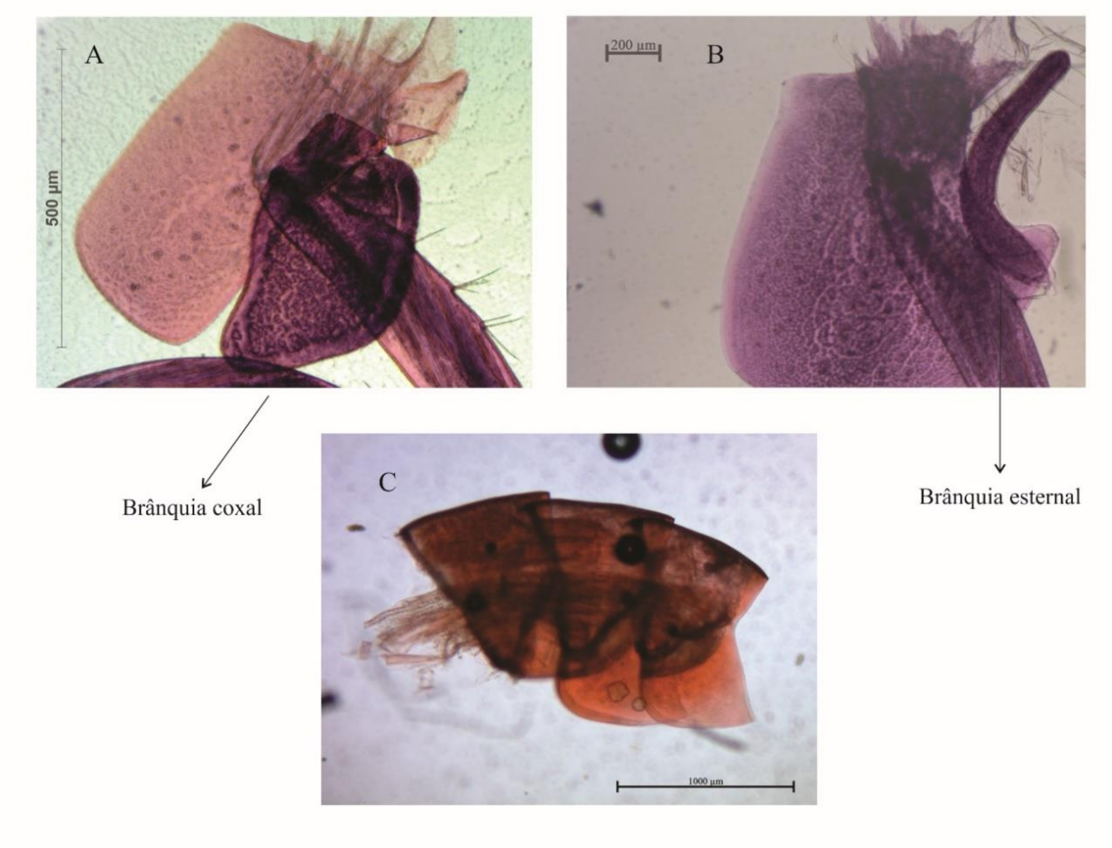


Fonte: Da autora (2023).

2.3 A influência do ambiente na morfologia dos animais

Um dos entendimentos mais básicos nos estudos sobre evolução e genética é que a atuação do ambiente no genótipo resulta no fenótipo. Em outras palavras, a forma com que os seres vivos se apresentam –ou seja: seu formato, tamanho, cor, etc., além de seu comportamento, são tanto resultado de componentes herdados geneticamente quanto das características ambientais onde o organismo vive.

Figura 9: Modelos de apêndices de *Hyalella* Smith, 1874. Foto de brânquia coxal (**A**); Foto de brânquia esternal (**B**); Foto de placas epimerais (**C**).



Fonte: Da autora (2023).

Além disso, as bases da teoria da evolução de Darwin (1859) nos dizem que os diferentes fenótipos estão sob constante pressão de seleção. Assim, quando comparamos a morfologia de diferentes grupos de animais, notamos que algumas características se repetem, mesmo em filos completamente diferentes, como resultado de evolução convergente (Begon *et al.*, 2009). Por exemplo, peixes e baleias possuem nadadeiras; e pássaros e insetos possuem asas; mas a origem das nadadeiras e das asas nesses pares de grupos não é a mesma, e sim um resultado da seleção natural em direção a um melhor *fitness* para os ambientes (água e ar, respectivamente) desses grupos. Ao contrário, filos evolutivamente próximos podem apresentar evolução divergente em alguma característica com resultado de diferentes pressões de seleção. É o caso das adaptações em formato de asa de morcegos, que são paralelas às mãos e dedos dos demais mamíferos (Begon *et al*., 2009).

A seleção natural também atua diferenciando as características de espécies mais relacionadas, por exemplo, dentro de um mesmo gênero. Nesse caso, é possível avaliar as respostas morfológicas que se repetem às mesmas pressões de seleção, aquelas que se diferenciam sob diferentes pressões, e aquelas que não são afetadas (Teixeira & Bennemann, 2007; Kirchheim & Goulart, 2010. Por exemplo, nos anfípodes do gênero *Niphargus* Schiödte, 1849, Trontelj *et al.* (2012) observaram uma relação direta entre tamanho do corpo e alongamento de apêndices com a velocidade da água e a disponibilidade de espaço (tamanho do poro), mostrando que diferentes espécies de *Niphargus* respondem de forma semelhante quando sob a mesma pressão ambiental. Para *Hyalella*, no entanto, não temos estudos que avaliam essa relação e de que forma o ambiente influencia na morfologia do gênero.

Por isso, essa tese tem como objetivo geral estudar a morfologia do gênero *Hyalella*; os objetivos específicos são: avaliar a influência de algumas variáveis ambientais na morfologia geral de espécies brasileiras de *Hyalella*; descrever cinco novas espécies do gênero; redescrever *Hyalella caeca* Pereira, 1989; estudar a filogenia de *Hyalella* através de análises moleculares de espécies troglóbias e epígeas encontradas nas regiões sul e sudeste do Brasil; e apresentar uma chave pictórica para identificação de espécies subterrâneas ou associadas ao ambiente subterrâneo de *Hyalella* brasileiras.

REFERÊNCIAS

BARNARD, J. L.; BARNARD, C. M. **Biogeographical microcosms of world freshwater Amphipoda (Crustacea).** Polskie Archiwum Hidrobiologii, v. 29(2), p. 255–273. 1982.

BEGON, M.; TOWNSEND, C. R.; HARPER, J. L. **Ecologia: de indivíduos a ecossistemas.** Artmed editora. 2009.

BRUSCA, R. C.; MOORE, W.; SHUSTER, S. M. **Invertebrados**, 3. ed. Grupo GEN. https://integrada.minhabiblioteca.com.br/books/9788527733458. 2018.

BUENO, A. A. P.; RODRIGUES, S. G.; ARAUJO, P. B. **O estado da arte do gênero *Hyalella* Smith, 1874 (Crustacea, Amphipoda, Senticaudata, Hyalellidae) no Brasil**. In: *HAYASHI, C. (Ed.). Tópicos de Atualização em Ciências Aquáticas*. Uberaba: UFMT, p. 57-88, 2014.

COPILAȘ-CIOCIANU, D.; SIDOROV, D.A.; GONTCHAROV, A.A. **Adrift across tectonic plates: molecular phylogenetics supports the ancient Laurasian origin of old limnic crangonyctid amphipods.** Organisms Diversity & Evolution, v. 19(2), p. 191-207, 2019. https://doi.org/10.1007/ s13127-019-00401-7

DARWIN, C. (1859). **Origem das espécies: ou A preservação das raças favorecidas na luta pela vida**. Ubu Editora LTDA-ME, 2018.

GALLÃO, J. E.; BICHUETTE, M. E. **Brazilian obligatory subterranean fauna and threats to the hypogean environment**. *ZooKeys*, v. 746, p. 1, 2018.

GONZÁLEZ, E. R.; BOND-BUCKUP, G.; ARAUJO, P. B. **Two new species of *Hyalella* from Southern Brazil (Amphipoda: Hyalellidae) with a taxonomic key**. Journal of Crustacean Biology, v. 26 (3), p. 355-365, 2006.

GROSSO, L. E.; PERALTA, M. **Anfípodos de agua dulce sudamericanos: revisión del género *Hyalella* Smith. I**. Acta Zoologica, v. 45(1), p. 79–89, 1999.

HANSEN, H.J. **The Ingolfiellidae, fam. n., a new type of Amphipoda.** Journal of the Linnean Society of London (Zoology), v. 29, p. 117–133, 1903. http://dx.doi.org/10.1111/j.1096-3642.1903.tb00430.x

KIRCHHEIM, P. D./ GOULART, E. **Ecomorphology of predation and antipredation in Siluriformes (Osteichthyes).** Oecologia Australis, v. 14(2), p. 550-568, 2010.

LIMBERGER, M.; SANTOS, S.; CASTIGLIONI, D. D. S. ***Hyalella luciae*** **(Crustacea, Amphipoda, Hyalellidae) – a new species of freshwater amphipod from Southern Brazil.** Zootaxa, v. 5174(5), p. 568-582, 2022.

LATREILLE, P.A. **Histoire Naturelle, générale et particulière des Crustacés et des Insectes**. v. 1–4. Paris, 1802.

LEACH, W.E. **Crustaceology**. The Edinburgh Encyclopaedia, v. 7, p. 383–434, 1814.

LOWRY, J. K./ MYERS, A. A. **A phylogeny and classification of the Senticaudata subord. nov. Crustacea: Amphipoda**. Zootaxa, v. 3610(1), p. 1-80, 2013.

MARRÓN-BECERRA, A.; HERMOSO-SALAZAR, M. **Morphological comparison and description of five new species of *Hyalella* (Crustacea: Amphipoda) from Veracruz and Mexico City.** Journal of Natural History, v. 56(25-28), p. 1215-1263, 2022.

MILNE EDWARDS, H.**Extrait de recherches pour server a l'histoire naturelle des crustaces amphipodes.** Annales des Sciences Naturelles, v. 20, p. 353–399, 1830.

PECHENIK, J. A. **Biologia dos Invertebrados**, 7. ed. Grupo A. 2016. https://integrada.minhabiblioteca.com.br/books/9788580555813

PENONI, L. R.; LARES, M. M. A.; BUENO, A. A. P. **Description of two new species of the freshwater amphipod *Hyalella* Smith, 1874 (Amphipoda: Hyalellidae) from southeastern Brazil, with remarks on their population biology and reproduction.** Journal of Crustacean Biology, v. 41(3), ruab050, 2021.

ROGERS, D. C.; MAGALHÃES, C.; PERALTA, M.; RIBEIRO, F. B.; BOND-BUCKUP, G.; PRICE, W. W.; ... SANTOS, S. **Phylum Arthropoda: Crustacea: Malacostraca.** In Thorp and Covich's Freshwater Invertebrates (pp. 809-986). Academic Press, 2020.

TEIXEIRA, I.; BENNEMANN, S. T. **Ecomorfologia refletindo a dieta dos peixes em um reservatório no sul do Brasil**. Biota Neotropica, v. 7(2), p. 67-76, 2007.

TRONTELJ, P./ BLEJEC, A./ FIŠER, C. **Ecomorphological convergence of cave communities.** Evolution: International Journal of Organic Evolution, v. 66(12), p.3852-3865, 2012.

VÄINÖLÄ, R.; WITT, J. D. S.; GRABOWSKI, M.; BRADBURY, J. H.; JAZDZEWSKI, K.; SKET, B. **Global diversity of amphipods (Amphipoda; Crustacea) in freshwater.** In Freshwater animal diversity assessment, Springer, Dordrecht, p. 241-255, 2007.

SEGUNDA PARTE

1. Capítulo 1

Is the environment important on the morphology of *Hyalella* Smith, 1874 (Amphipoda: Hyalellidae)?

Artigo preparado para submissão para o periódico Zoosystematics and Evolution

Is the environment important on the morphology of *Hyalella* Smith, 1874 (Amphipoda: Hyalellidae)?

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Abstract

Hyalella is a freshwater amphipod genus greatly distributed on the South and Southeast regions of Brazil. The morphology within the genus, especially among the Brazilian representatives of the genus, is well preserved, with many species similar to each other, and there is no research about the environmental variables affecting the morphology. Here we discuss the importance of the environment on the morphological variations of *Hyalella*. We constructed models to evaluate the influence of eight environmental variables on 21 morphological measurements. Our results indicate that the minimum annual temperature was the most important abiotic variable affecting the morphology of *Hyalella*, followed by the epigean *x* hypogean habit. Interestingly, there was no impact of the microhabitat association preference on the morphology of the studied species. We also found that in general the species are very similar, with no formation of groups based on morphology, and only a few species separated from the others.

Key words: Abiotic variables; Freshwater amphipods; Morphology.

Introduction

Hyalella is an Amphipod genus exclusive to the American continent and widely distributed on the South and Southeast geopolitical regions of Brazil (Bueno et al., 2014; Rangel et al., 2022). Many new Brazilian species of this crustacean were described in the past few years as a result of the establishment of new research groups for the genus, material collections at new regions and areas, and the analysis of material deposited at zoological collections (Reis et al., 2020; Penoni et al., 2021; Limberger et al., 2022; Rangel et al., 2022). With all this new information it was possible to observe that many *Hyalella* species are endemic to their type locality; also, that the morphology within the genus is well preserved with morphologically similar species (Bueno et al., 2014; Rangel et al., 2022).

In Brazil, *Hyalella* species occur on many different freshwater environments, including springs, ponds, streams, wetlands/flooded areas, caves, and others (Bueno et al., 2014; Cardoso et al., 2014; Rangel et al., 2022). Some species are associated to lentic habitats, others to lotic ones (Penoni et al., 2021); the same way, some *Hyalella* preferably associate to rocky substrates (Penoni et al., 2021), others to sandy bottoms , others swim freely on the water current (Cardoso et al., 2014), ant there are those which are associated to the water vegetation (Talhaferro et al., 2021); and finally, some species are epigean (superficial), and other are hypogean (subterranean) (Cardoso et al., 2014). It is also important to consider that there are species known to four different biomes: the Pampa, the Atlantic Rainforest, the Cerrado (Brazilian Savanna) and the Caatinga (Brazilian dry tropical forest) (Bueno et al., 2014). It implies different hydric cycles and annual temperatures on the *Hyalella* species of Brazil. Yet, there is great morphological similarity within the species of the genus.

The process of describing new *Hyalella* species require the details about structures general format and size, cuticular and ultra-cuticular sensory apparatus – *i. e.* setae and setules

–, somite formats, and gills distribution (González & Coleman, 2002). There is no specific characteristic that can be used to identify an *Hyalella* species: you need a group of features to show that one species is different from another. Nevertheless, we need to account for intraspecific variations and remember that a species is a hypothesis (Lukhtanov, 2019), and a holotype is only a representative of that species.

Additionally to the intrinsic components of a species, the environment should be accounted when determining morphology. It means that biotic and abiotic variables, such as temperature, water current and predators can play important roles on the phenotypes exhibit by a population (Kruschwitz, 1978; Wellborn & Cothran, 2007; Trontelj et al., 2012). For example, sympatric North American *Hyalella* species have shown a tradeoff between competitive ability and antipredator adaptation (Wellborn & Cothran, 2007). The reduction of mean body size lead to a reduced risk of predation while a greater body size, although directly related to better competitive power, lead to an increased chance of being seen by fish and so to be predated (Wellborn, 2002).

Here we explore the influence of the environment on some of the morphological aspects of *Hyalella* and discuss which environmental actors play important roles on the morphology of the genus.

Materials and methods

Data sampling

To understand the importance of some environmental variables on the morphology of *Hyalella,* we used *Hyalella* specimens from 18 different species. All but two are isolated (do not co-occur with other *Hyalella* species): *H. bala* Penoni & Bueno, 2020 and *H. virgineae* Lares, Penoni and Bueno, 2020 live in sympatry (Penoni et al., 2020). Table S1 presents detailed information on species environmental aspects. This material was available at the Crustacean

Collection of Universidade Federal de Lavras (CCUFLA), and the environmental data was obtained from personal observation of the collectors (altitude; epigean *x* hypogean habit; microhabitat association preference; lentic *x* lotic habit; and coexistence with other *Hyalella* species) and the values of maximum and minimum temperatures were obtained from the Clima Tempo website database, available at https://www.climatempo.com.br/climatologia.

For morphological analysis we prepared semi-permanent slides of 7–12 male adult specimens of each species. We stained the selected animals with the "red Congo" die for 24–36h for better contrast and detailed observation. Before dissection, we measured the total body length of each specimen from head (starting at the insertion of the antenna 1) to just before the telson. Dissection was performed under a stereomicroscope with the aid of fine needles. The appendages where carefully placed on slides with glycerin, covered with a coverslip and sealed with transparent painting varnish.

We transferred the slides to a Zeiss Primo Star microscope with an attached Zeiss AxioCam ERc5s camera and photographed and measured the body components using the Zen 2011 software. These are the 21 morphometric variables that we observed: body length (1); antenna 1 total length (2); antenna 1 total length *x* antenna 2 peduncle length ratio (3); antenna 2 mean number of articles on the flagellum (4); antenna 2 total length (5); gnathopods 1 (6) and 2 (7) and pereopods 3 to 7 (8–12) coxae length *x* width ratio; pereopods 3–7 (13–17) total length*;* uropods 1 to 3 (18–20) total length; and uropod 3 peduncle length *x* ramus length ratio (21). Missing data (any individual measurement that was not possible to be performed due to missing appendages) was replaced by the means of the species.

Data analysis

We evaluated the effects of the eight environmental variables (altitude; epigean *x* hypogean habit; biome; maximum annual temperature; minimum annual temperature;

microhabitat association preference; lentic *x* lotic habit; and coexistence with other *Hyalella* species) on the 21 morphological measurements. The categorial variables options were noted as follows: epigean – yes/ no; biome – Pampa/ Atlantic Rainforest/ Cerrado; microhabitat association preference – substrate/ vegetation/ pluriestratified/ emergent; lentic – yes/ no; and coexistence – yes/ no. Before modelling, the continuous variables (altitude, annual minimum and maximum temperature) were scaled by centering with zero as so to obtain comparable coefficients.

To evaluate the effects of environmental variables on morphological attributes, we used linear mixed models (LMMs) with *gaussian* family, using Species as a random factor to control for the dependence between measurements performed in the individuals of the same species, also considering the assumptions of homoscedasticity and residuals normality. We first built a global model for each response variable in the template (1).

$$y \sim altitude + epixhipo + biome + maximum\ annual\ temperature + minimum\ annual\ temperature + microhabitat\ association\ preference + lenticxlotic + coexistence + (1|Species) \qquad (1)$$

From the global model, we obtained all submodels for each morphological response variable with possible combinations of noncorrelated variables ($r <| 0.6 |$) using the *dredge* function of the *MuMIn* package (Barton, 2020). Best models were thus selected based on an AICc (Akaike Information Criterion of second order) delta of 4 (ΔAICc = 4, Burhnham et al., 2011). These best submodels were subjected to multi-model inference (Burnham et al., 2011) using the *model.avg* function of the *MuMIn* package (Barton, 2020), to obtain the average coefficients of the explanatory variables and their significance. For categorical variables with significative effects, we performed a post-hoc test to identify differences between categories, using the Least-Squares means test (Ls-means) (Lenth, 2020) at a significance level of 5%.

Species morphological groups

To visualize the formation species groups based on the morphological variables, we performed a Principal Component Analysis (PCA) at individual level using the *prcomp* function of the *stats* package. Lastly, we produced a Non-metric Multi-dimensional Scaling (NMDS) ordination to represent the species patterns between plots, using Euclidean as distance measure, applied on scaled variables to avoid the influence of measurement units. We performed all analysis in the R program (R Core Team, 2020).

Results

We observed that, amongst the environmental variables that we studied, the minimum annual temperature is the one that affects more morphological variables (14 out of 21), and surprisingly the microhabitat association preference showed no influence on the morphology of *Hyalella*. Table 1 summarizes the submodels results.

The minimum annual temperature had a significant negative effect on the body length, on the antenna 1 length *x* antenna 2 peduncle ratio, on the antenna 2 length, on the length *x* width ratios of coxae 1–4, and on the total lengths of pereopods 3, 5–7 and uropod 1. It also had a positive effect on the length *x* width ratios of coxae 5 and 6. The other continuous environmental variables had effect only on the gnathopod 1 coxa length *x* width ratio: the altitude had a negative and the maximum annual temperature had a positive effect on it.

For the categorical environmental variables, the epigean *x* hypogean habit affected nine out of 21 morphological variables, followed by coexistence with other *Hyalella* species, which affected five variables; the lentic *x* lotic habit affected three morphological variables; and finally the biome affected two morphological variables. Table 2 summarizes the effects of the categorical variables.

Species morphological groups

PCA performed on all individuals of the 18 *Hyalella* species showed no tendency to any group formation based on the morphological variables, with individuals of many different species altogether in the middle of the plot (Fig. 1). Yet, we see some species with individuals grouped on the ends of the plot, such as the *Hyalella bala*, *H. kaingang* and *H. imbya* individuals, each of which on a different quadrant. Principle Component 1 (PC1 – x axis) explained 47.27% of the variation and PC2 (y axis) explained 19.75%, with combined explanation of 67.02% of data variation. Table S2 details de variables' contributions to each component. Similarly to the PCA, the NMDS shows the grouping of individuals of *Hyalella bala*, *H. kaingang* and *H. imbya*, with individuals of the other species scattered on the middle of the plot (Fig. 2).

Discussion

This research is the first to present the influence of environmental factors on the general morphology of *Hyalella* from Brazil. Here we observed that the temperature and the epigean *x* hypogean habit influence the general morphology of the studied species. The Brazilian hyallelids, in general, present a quite conserved morphology, with only a few subtle differences between species. That means that the descriptions of new species and species identifications are based on several morphological details, because there is no specific characteristic that can be used during the identification process.

Here we saw that the minimum annual temperature was the environmental factor that affected most of the morphological response variables, including the ratio between length and width of the coxae 1–6 (with negative effects on coxae 1–4, and positive on coxae 5 and 6). These structures are directly related to oxygenation of the gills, since the coxae, together with the epimeral plates, form a longitudinal ventral channel (Trontelj et al., 2012). In *Hyalella*, the anterior lobe of coxa 6 and the coxa 7 are reduced, and coxae 1–4 are larger, acting as a barrier to this longitudinal channel. Our results suggest that the species from regions with colder minimum annual temperatures have coxae 1–4 more squared than those from places with greater minimum annual temperature, as so in warmer temperatures, the channel gets longer. In general, amphipods have low tolerance to hypoxia and are more adapted to colder waters (Väinola et al., 2008; Copilaş-Ciocianu et al., 2019, 2020), and a longer ventral channel could lead to greater oxygen intake on warmer waters.

We also saw a great impact of the epigean *x* hypogean habit in general. This one, although classical and important, needs to be more carefully discussed in our study, since we only had one hypogean representative, *Hyalella imbya*. Subterranean *Hyalella*, specially the cave species, usually have really small populations and it is hard to collect a great number of individuals. Because of that, we did not have access to enough hypogean *Hyalella* populations with a good enough N to be included here. Yet, we saw that *H. imbya* was isolated on both PCA and NMDS plots, and their morphological measurements were significantly different from the epigean species, with antenna 1 four times longer than the peduncle of antenna 2 (while for epigean species antenna 1 was twice as long as the peduncle of antenna 2, in average) and the uropods of *H. imbya* are shorter than the uropods, in general, of epigean species. The classical troglomorphisms (adaptations to the subterranean life) include the elongation of structures (Culver et al., 1995), which was observed for the antennas of *H. imbya*.

Two results that were unexpected are the absence of effects of the microhabitat association preference and the low importance of the lentic *x* lotic habit, which affected only the length *x* width ratio of coxae 1–3. Previous studies with another amphipod genus, the *Niphargus*, clearly indicated the importance of these habits on the general morphology of niphargids, even with the formation of morphological groups as a response to the microhabitat (Trontelj et al., 2012). It is worth noting that *Hyalella* are usually endemic, and many Brazilian species do not coexist with other *Hyalella*, while *Niphargus* form communities of many species. This results in intrageneric competition, which leads to niche divergency (Wellborn & Cothran, 2007; Trontelj et al., 2012). For *Hyalella*, this was observed in North American sympatric species, with direct impacts on body length and behavioral aspects (Wellborn & Cothran, 2007).

Finally, we saw on the PCA and NMDS plots that species don’t form morphological groups, and for most cases, the individuals of many different species share a great deal of morphological information, with only a few species clearly separated from the others. This resonates with the fact that, when identifying *Hyalella* or describing a new species of the genus, one needs attention to details and to stablish which set of characteristics can be used to properly identify a species.

Acknowledgements

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References

Barton, K. (2020). MuMIn: multi-Model Inference. R package version 1.42.1. Available at https://cran.r-project.org/package=MuMIn

Bueno, A. A. P., Rodrigues, S. G. & Araujo, P. B. (2014). O estado da arte do gênero *Hyalella* Smith, 1874 (Crustacea, Amphipoda, Senticaudata, Hyalellidae) no Brasil. In: *HAYASHI, C. (Ed.). Tópicos de Atualização em Ciências Aquáticas*. Uberaba: UFMT, 2014. p. 57-88.

Burnham, K.P., Anderson, D.R., & Huyvaert, K.P. (2011). AIC model selection and multimodel inference in behavioral ecology: some background, observations, and comparisons. *Behavioral Ecology Sociobiology, 65* (1), 23–35. https://doi.org/10.1007/pnas00265-010-1029-6.

Cardoso, G. M., Araujo, P. B., Bueno, A. A. P., & Ferreira, R. (2014). Two new subterranean species of *Hyalella* Smith, 1874 (Crustacea: Amphipoda: Hyalellidae) from Brazil. *Zootaxa*, *3814*(3), 353-368.

Culver, D. C., Kane, T. C., & Fong, D. W. (1995). Adaptation and natural selection in caves: the evolution of *Gammarus minus*. Harvard Univ. Press, London.

González, E. R., Coleman, C. O. (2002). *Hyalella armata* (Crustacea, Amphipoda, Hyalellidae) and the description of a related new species from Lake Titicaca. *Organisms Diversity & Evolution*, *6*, 1–19.

Kruschwitz, L. G. (1978). Environmental Factors Controlling Reproduction of the Amphipod *Hyalella azteca*. In *Proceedings of the Oklahoma Academy of Science* (pp. 16-21).

Lenth, R. (2020). Emmeans: Estimated Marginal Means, aka Least-Squares Means. R package version 1.4.6. https://CRAN.R-project.org/package=emmeans

Limberger, M., Santos, S., & Castiglioni, D. D. S. (2022). *Hyalella luciae* (Crustacea, Amphipoda, Hyalellidae) – a new species of freshwater amphipod from Southern Brazil. *Zootaxa*, *5174*(5), 568-582.

Lukhtanov, V. A. (2019). Species delimitation and analysis of cryptic species diversity in the XXI century. *Entomological Review, 99(4)*, 463-472.

Penoni, L. R., Lares, M. M. A., & Bueno, A. A. P. (2021). Description of two new species of the freshwater amphipod *Hyalella* Smith, 1874 (Amphipoda: Hyalellidae) from southeastern

Brazil, with remarks on their population biology and reproduction. *Journal of Crustacean Biology, 41*(3), ruab050.

R. Core Team. (2020). *R: a Language and Environment for Statistical Computing. R Foundation for Statistical Computing*. Available at http://www.R-project.org

Rangel, C., Silva, A. L. L., Siegloch, A. E., & Limberger, M. Castiglioni, D. D. S. (2022). First island species of *Hyalella* (Amphipoda, Hyalellidae) from Florianpolis, state of Santa Catarina, Southern Brazil. *Zootaxa, 5116*(1), 40-60.

Reis, G. O., Penoni, L. R., & Bueno, A. A. (2020). First record of the genus *Hyalella* (Amphipoda: Hyalellidae) from Santa Catarina State, Brazil, with description of two new species. *Biota Neotropica, 20*.

Talhaferro, J. T., Bueno, A. A. P., Pires, M. M., Stenert, C., Maltchik, L. & Kotzian, C. B. (2021). Three new species of *Hyalella* (Crustacea: Amphipoda: Hyalellidae) from the Southern Brazilian Coastal Plain. *Zootaxa*, 4970(2), 257292.

Trontelj, P., Blejec, A., & Fišer, C. (2012). Ecomorphological convergence of cave communities. Evolution: *International Journal of Organic Evolution, 66*(12), 3852-3865.

Wellborn, G. A. (2002). Trade-off between competitive ability and antipredator adaptation in a freshwater amphipod species complex. *Ecology, 83*(1), 129-136.

Wellborn, G. A., & Cothran, R. D. (2007). Niche diversity in crustacean cryptic species: complementarity in spatial distribution and predation risk. *Oecologia, 154*(1), 175-183.

Tables:

Table 1: Effect of environmental variables on structure measurements of *Hyalella* from Brazil. Blue squares represent significant effect for the categorical variables on the morphological measurements; pink squares represent significant negative effect for the continuous variables; and green squares represent significant positive effect for the continuous variables. Values represent the estimate coefficient of the submodel. "x" means no effect.

	Epigean *x* Hypogean habit	Biome	Microhabitat association preference	Lentic *x* Lotic habit	Coexistance	Altitude	Maximum annual temperature	Minimum annual temperature
Body length	x	x	x	x	x	x	x	-0.08378
A1 length	x	x	x	x	x	x	x	x
A1 length *x* A2 peduncle	🟦	🟦	x	x	x	x	x	-0.09659
A2 #articles	x	🟦	x	x	x	x	x	x
A2 length	x	x	x	x	x	x	x	-0.10994
G1 coxa length *x* width	x	x	x	🟦	🟦	-0.0207	0.019084	-0.02502
G2 coxa length *x* width	🟦	x	x	🟦	🟦	x	x	-0.03387
P3 coxa length *x* width	🟦	x	x	🟦	🟦	x	-0.03707	-0.04841
P4 coxa length *x* width	x	x	x	x	x	x	x	-0.02106
P5 coxa length *x* width	🟦	x	x	x	🟦	x	x	0.020905
P6 coxa length *x* width	🟦	x	x	x	🟦	x	x	0.04608
P7 coxa length *x* width	🟦	x	x	x	x	x	x	x
P3 length	x	x	x	x	x	x	x	-0.11453
P4 length	x	x	x	x	x	x	x	x
P5 length	x	x	x	x	x	x	x	-0.09533
P6 length	x	x	x	x	x	x	x	-0.11954
P7 length	x	x	x	x	x	x	x	-0.11678
U1 length	🟦	x	x	x	x	x	x	-0.10617
U2 length	🟦	x	x	x	x	x	x	x
U3 length	🟦	x	x	x	x	x	x	x
U3 peduncle *x* ramus	x	x	x	x	x	x	x	x

Table 2: Summary of the effect of categorical environmental variables on the morphological variables of *Hyalella*. Numbers represent the mean value of the variable to each category. Letters "a" and "b" indicate the difference between categories. "x" means no effect.

	Biome			Epigean *x* hypogean habit		Lentic *x* lotic habit		Coexists with other *Hyalella*	
A1 length *x* A2 peduncle	Cerrado 3.72a	Atlantic Rainforest 2.85b	Pampa 2.74b	Epigean 2.13b	Hypogean 4.07a	x		x	
A2 #articles	Atlantic Rainforest 14.939a	Pampa 14.113ab	Cerrado 13.000b	x		x		x	
G1 coxa length *x* width	x			x		Lentic 0.617a	Lotic 0.575b	Coex no 0.622a	Coex yes 0.570b
G2 coxa length *x* width	x			Epigean 0.571b	Hypogean 0.737a	Lentic 0.676a	Lotic 0.632b	Coex no 0.697a	Coex yes 0.611b
P3 coxa length *x* width	x			Epigean 0.599b	Hypogean 0.710a	Lentic 0.684a	Lotic 0.625b	Coex no 0.701a	Coex yes 0.608b
P5 coxa length *x* width	x			Epigean 0.705a	Hypogean 0.558b	x		Coex no 0.607b	Coex yes 0.647a
P6 coxa length *x* width	x			Epigean 1.039a	Hypogean 0.892b	x		Coex no 1.011795b	Coex yes 1.149133a
P7 coxa length *x* width	x			Epigean 0.688a	Hypogean 0.542b	x		x	
U1 length	x			Epigean 1321a	Hypogean 807b	x		x	
U2 length	x			Epigean 836a	Hypogean 526b	x		x	
U3 length	x			Epigean 414a	Hypogean 197b	x		x	

Figures:

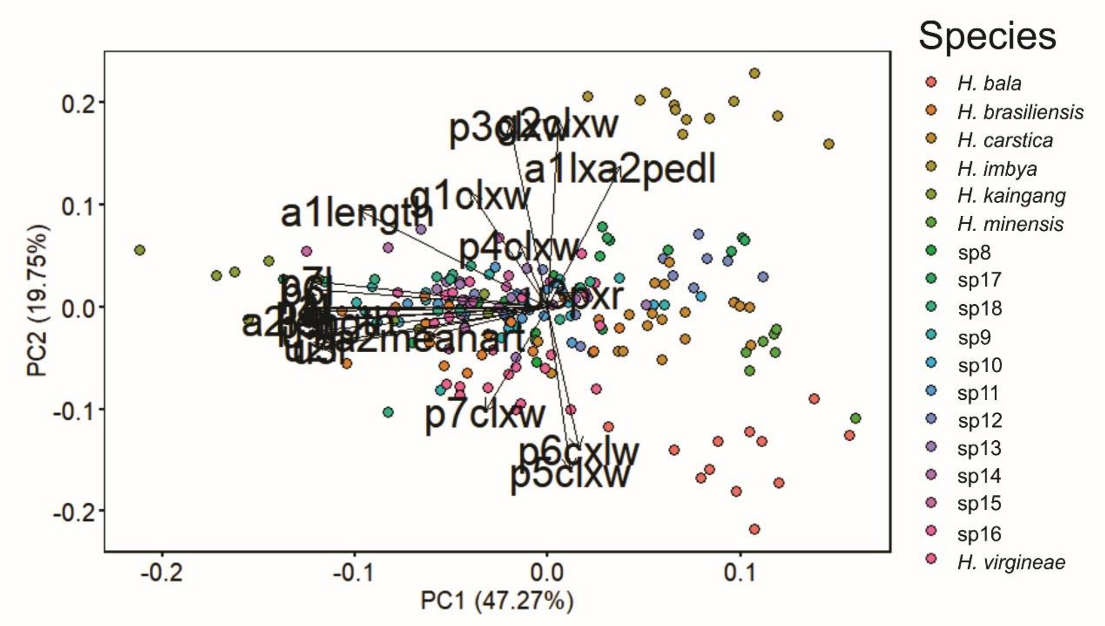

Figure 1: Principal Component Analysis (PCA) plot for 18 *Hyalella* species based on morphological measurements. See table S2 for principal components and morphological variables correlations.

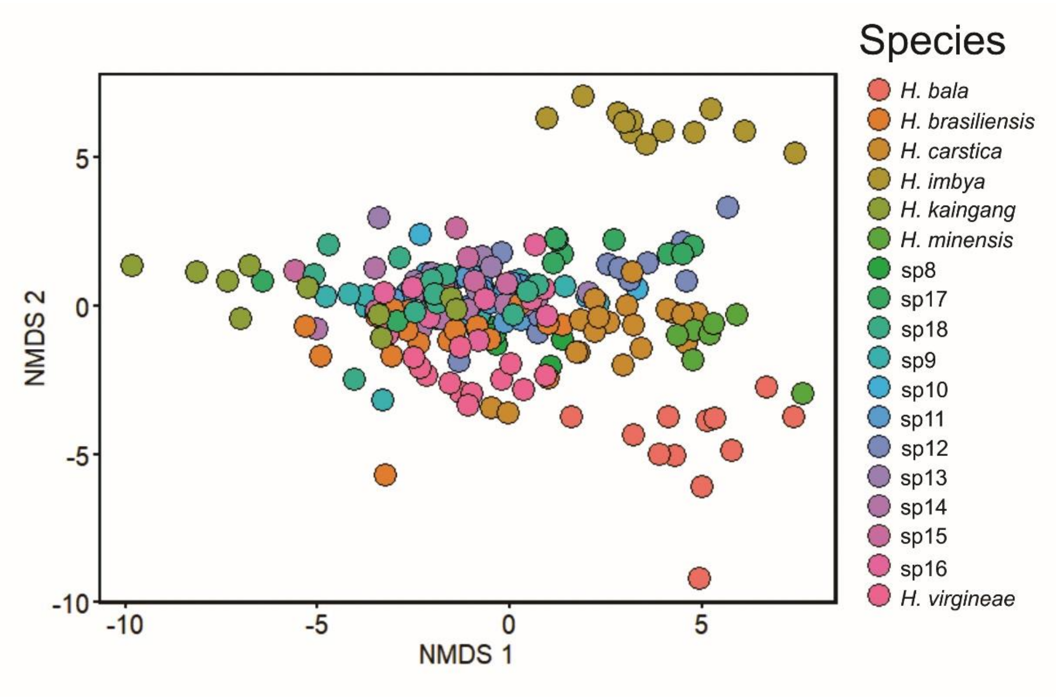

Figure 2: Non-metric multidimensional scaling (NMDS) plot for 18 *Hyalella* species based on morphological measurements.

Supplementary material

Table S1: Collection sites environmental variables for *Hyalella*. Labels: Lote – scientific collection number (from Coleção de Crustáceos da Universidade Federal de Lavras – CCUFLA); alt – altitude, in meters; epig – epigean (y – yes) *x* hypogean (n – no); tmax – maximum annual temperature, in degrees Celsius; tmin – minimum annual temperature, in degrees Celsius; assoc. – microhabitat association preference (subs – substrate; veg – aquatic vegetation; pluri – pluriestratified; emer – emergent); lentic – lentic (y – yes) *x* lotic (n – no); coex – coexistence with other *Hyalella* species (y – yes; n – no).

Species	**Lote**	**City/State**	**Latitude/ Longitude**	**alt**	**epig**	**bioma**	**tmax**	**tmin**	**assoc.**	**lentic**	**coex**
sp8	188	Santa Vitória do Palmar/RS	33º06'18.7"S 52º55'08.8"w	33	y	pampa	26	9	veg	y	n
sp9	190	Cachoeira do Sul/RS	30º16'08.2"S 52º57'42.1"W	106	y	pampa	31	9	veg	y	n
sp10	192	Armada/RS	30º43'34.7"S 55º08'40.4"W	184	y	pampa	28	8	veg	y	n
sp11	193	Pedro Osório/RS	31º51'31.4S 52º48'48.5"W	4	y	pampa	28	9	pluri	y	n
sp12	195	Tupanciretã/RS	29º03'04.3"S 53º43'20.9"W	445	y	atlantic	29	9	emer	y	n
sp13	197	Erechin/RS	27º35'39.1"S 52º10'23.7"W	666	y	atlantic	28	8	emer	y	n
sp14	198	Santo Cristo/RS	27º50'05.8"S 54º36'50.3"W	216	y	atlantic	31	11	emer	y	n
sp15	24	São José dos Ausentes/RS	28º49’43"S 50º00’04"W	900	y	atlantic	24	8	emer	n	n
sp16	250	Bagé/RS	30º47'34,2"S 53º34'44,6"W	214	y	pampa	29	8	veg	y	n
sp17	251	Canguçu/RS	31º02'41,1"S 52º43'26,5"W	118	y	pampa	28	8	emer	y	n
sp18	252	Porto Xavier/RS	27º57'37"S 55º07'51"W	267	y	atlantic	32	11	emer	y	n
bala	443	São Luiz do Paraitinga/SP	23º19’24,5”S 45º07’53,5W	912	y	atlantic	27	13	subs	n	y
brasiliensis	96	Piraí do Sul/PR	24º30’5,3”S 49º5358,5”W	839	y	atlantic	26	10	subs	n	n
carstica	258	Arcos/MG	20º20‘‘01,5”S 45º36‘‘40,7”W	706	y	cerrado	28	13	veg	n	n
imbya	187	Roque González/RS	28º13'55.6S 54º58'37.3W	203	n	atlantic	32	11	emer	y	n
kaingang	121	São Francisco de Paula/RS	29º12’35,8”S 50º14’22”W	915	y	atlantic	26	8	emer	n	n
minensis	253	Lavras/MG	21°13'84"S, 44°58'66"W	925	y	atlantic	28	12	subs	n	n
virgineae	445	São Luiz do Paraitinga/SP	23º19’36,5”S 45º08”01,1”W	915	y	atlantic	27	13	emer	y	y

Table S2: Principal component analysis (PCA) summary table. Principal components 1 and 2 combined explain 67.02% of variations. Legend: bl – body length; a1length – antenna 1 total length; a1lxa2ped – antenna 1 total length *x* antenna 2 peduncle length ratio; a2meanart – antenna 2 mean number of articles on the flagellum; a2length – antenna 2 total length; g – gnathopod; p – pereopod; clxw – coxa length *x* width ratio; l – length; u – uropod; u3pxr – uropod 3 peduncle length *x* ramus length ratio.

Variable	Component 1	Component 2
bl	-0.28748546	-0.009406383
a1length	-0.23729111	0.22936099
a1lxa2pedl	0.09077718	0.335590868
a2meanart	-0.14769716	-0.06548149
a2length	-0.2867389	-0.027943117
g1clxw	-0.09672275	0.270110492
g2clxw	0.01306596	0.442410909
p3clxw	-0.04745977	0.432199746
p4clxw	-0.03579002	0.149270823
p5clxw	0.02768046	-0.38669331
p6clxw	0.03823236	-0.336379317
p7clxw	-0.07856175	-0.245057602
p3l	-0.30782842	-0.003928797
p4l	-0.30725561	-0.001540686
p5l	-0.30608937	-0.034468371
p6l	-0.3023083	0.041870023
p7l	-0.30346759	0.062376967
u1l	-0.29946678	-0.059057068
u2l	-0.29683597	-0.090582152
u3l	-0.28645685	-0.096669172
u3pxr	0.03352994	0.035014959

2. Capítulo 2

Artigo: A new species of *Hyalella* Smith, 1874 (Malacostraca: Amphipoda: Hyalellidae), and a DNA-based phylogeny reconstruction for cave-related *Hyalella* species from Southeast Brazil

Artigo preparado para submissão para o periódico Zoosystematics and Evolution

A new species of *Hyalella* Smith, 1874 (Malacostraca: Amphipoda: Hyalellidae) and a DNA-based phylogeny reconstruction for cave-related *Hyalella* species from Southeast Brazil.

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Abstract

Hyalella is a freshwater amphipod genus widely distributed in the American continent. Brazilian species of the genus present a relatively conserved morphology, and the identification of species require a detailed observation of structures. A phylogeny reconstructed based on DNA can help on the species identification. Here we describe a new species of the genus and present a phylogenetic reconstruction for cave-related *Hyalella* species. The new species has maxilla 1 palp relatively long, male gnathopod 2 palm with a minute posterior excavation, and uropod 3 longer than the telson, with peduncle and ramus subequal in length.

Key words: Descriptive taxonomy, hypogean amphipod, molecular phylogeny, troglophile invertebrate.

Introduction

The past decade represents a period of improvement on our knowledge of the genus *Hyalella* Smith, 1874. There was an increment of 36 new species of the genus, which represents almost 40% of the known *Hyalella* (Penoni et al., 2021; Limberger et al., 2022; Rangel et al., 2022). Yet, many new species are constantly being discovered, and there is a great potential for research with this genus. Populations of *Hyalella* can be found on the most variable freshwater habitats of the American continent, where the genus is restricted to, and Brazil is home to almost half of the known species (Penoni et al., 2021; Limberger et al., 2022; Rangel et al., 2022).

The description process of *Hyalella* requires a detailed examination of appendages and cuticular components –the setae (Zimmer et al., 2009), and there is no specific structure that allows one to identify a species (Bueno et al., 2014). Although there is great morphological diversity within the genus (Jurado-Rivera et al., 2020), there is also great similarities between species (González, 2003), with some classical misidentifications and "species complexes" such as the *Hyalella azteca* and the *Hyalella curvispina* complexes (González & Watling, 2002; González, 2003; Bueno et al., 2014; Jurado-Rivera et al., 2020).

The advent of molecular analysis allowed a new perspective for the phylogenetic studies (Lukhtanov, 2019). It can be a powerful tool helping understand the relationships between species within a genus (Jurado-Rivera et al., 2017, 2020; Zapelloni et al., 2021). For example, the species flock of *Hyalella* from Lake Titicaca presents a great number of morphologically similar species due to evolutive convergence, and it was only possible to evaluate the intricate phylogenetic relationships with the aid of molecular tools (Jurado-Riveira et al., 2020).

Here we describe a new species of *Hyalella* from Southeast Brazil, specifically a region within the state of São Paulo with many newly discovered species. We also use two DNA genes to reconstruct the phylogeny of Brazilian cave related *Hyalella* species.

Materials and methods

New species morphological description

A new species of *Hyalella* was collected in the "Gruta da Água Sumida", a cave from Parque Estadual Turístico do Alto Ribeira (PETAR), municipality of Iporanga, state of São Paulo, Brazil (Fig. 1). Collections were authorized by SISBIO/ICMBio under permission 28992 and by Instituto Florestal-SP under permission 002914/2121-12. *Hyalella* individuals were collected with the aid of fine hand nets and plastic pipettes, and the animals were stored in labeled flasks with ethyl alcohol 70%.

For the morphological description, two males were dissected and mounted on slides. They were measured for the total length (from head to lest segment before the telson) and head length and stained with Rose Bengal stain for 16–24 hours. Dissection was executed under a stereomicroscope and the appendages were mounted on slides with glycerin.

We photographed the appendages, and the pictures were used as base for structures' drawing, using the CorelDRAW X7 software. The description of appendages and sensory structures followed Zimmer et al. (2009) and Rogers et al. (2020).

Type material was deposited at the Crustacean Collection of Museu Nacional do Rio de Janeiro, municipalityy and state of Rio de Janeiro, Brazil (MNRJcarcino), Coleção de Crustáceos do Laboratório de Carcinologia da UFLA, municipality of Lavras, state of Minas Gerais, Brazil (CCUFLA), and the Zoological Collection of the Laboratório de Estudos Subterrâneos, Universidade Federal de São Carlos, municipality of São Carlos, state of São Paulo, Brazil (LES).

Molecular analysis - Extraction

We reconstructed the molecular phylogeny of some species of cave-related Brazilian *Hyalella*. Specimens of 11 species were used in this analysis. Some of the material was donated by partner researchers and some were collected for the development of this research. Specimens were preserved either on 70% or 100% ethanol.

We used 1 – 3 individuals of the following species: *Hyalella* sp6., municipality of Nobres, state of Mato Grosso; *Hyalella* sp7., from Fendão cave, municipality of Iporanga, state of São Paulo (PEI); *Hyalella sp1* n. sp., from Água Sumida cave, municipality of Iporanga, state of São Paulo (PETAR); *H. bala* Penoni and Bueno, 2021, municipality of São Luís do Paraitinga, state of São Paulo (Penoni et al., 2021); *H. bocaina* Deotii, Penoni and Bueno (in press), municipality of Ribeirão Grande, state of São Paulo (Deotti et al., in press); *H. sp4* Penoni and Bueno (in press), municipality of Eldorado, state of São Paulo (Penoni et al., in press – B); *H. sp3* Penoni and Bueno (in press), municipality of Iporanga, state of São Paulo (PETAR) (Penoni et al., in press – A); *H. sp2* Penoni and Bueno (in press), Santana cave, municipality of Iporanga, state of São Paulo (PETAR) (Penoni et al., in press – A); *H. temimina* Penoni, Deotti and Bueno (in press), Temimina II cave, municipality of Apiaí, state of São Paulo (PETAR) (Deotti et al., in press); *H. veredae* Cardoso and Bueno, 2014, caves “Zé de Sidney” and “Lapa de São Bernardo”, municipality of Presidente Olegário, state of Minas Gerais (Cardoso et al., 2014);

and *H. virgineae* Lares, Penoni and Bueno, 2021, municipality of São Luís do Paraitinga, state of São Paulo (Penoni et al., 2021).

DNA was extracted from muscular tissue using the Chelating ion-exchange Resin (Chelex VR 100) (Estoup et al., 1996). Selected animals were placed on sterile parafilm and dissected under a stereomicroscope, where the stomachs were separated and discarded. The remaining tissue was stored on identified plastic tubes and placed on the dry bath at 55 ºC for 3 minutes for the remaining alcohol to evaporate completely. After that, 200 µL of the Chelex-100 solution was added to each tube, which were agitated and homogenized before tissue maceration with sterile plastic bars. We added 10 µL of refrigerated Proteinase K (20 mg/ml) to each tube, agitated and placed the tubes on the dry bath at 55 ºC for 24 hours. On the next day, the dry bath temperature was increased to 95 ºC for 3 minutes, and the samples were transferred to the freezer for 10 minutes. Samples were centrifuged at 14000 rpm at 18 ºC for 3 minutes and the Chelex-100 solution was carefully removed from the tubes. The samples were then ready to be used for PCR.

Molecular analysis – PCR (Polymerase Chain Reaction)

For Polymerase Chain Reaction (PCR) (Sambrook et al., 1989), we added 1 – 5µL of DNA sample to a plastic tube with 5 µL of betaine, 4 µL of DNTPs (deoxynucleotide triphosphates), 3 µL of PCR buffer, 3 µL of $MgCl_2$, 2 µL of BSA 1% and 1µL of each primer. We used two sets of primers: one for a region of the mitochondrial gene 16S (16Samphi-f: 5'-AGTGYACAAATTGCCCGTCAYTCTC-3'; 16Samphi-r: 5'-ACAADTTATTATGCTACCTTAGCA C-3') (Rodrigues, 2016); and other for the nuclear 18S gene (18SO: 5′- AAGGGCACCACCAGGAGTGGAG-3′; 18SB: 5′-TGATCCTTCCGCAGGTTCACCT-3′) (Medlin et al. 1988).

PCR procedures were executed using the Applied Biosystems thermocycler, with initialization at 95 ºC, 40 cycles of 30 seconds at 95 ºC for denaturation, 30 seconds at 57.5 ºC for annealing, 1 minute at 72 ºC for extension, and finally 10 minutes at 72 ºC for final holding. Efficiency of PCR was observed through electrophoresis and successful samples were sent for Sanger sequencing, with Big Dye® Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems) at the automatic sequencer ABI3730xl Genetic Analyzer (Applied Biosystems)® by the Empresa Biotecnologia Pesquisa e Inovação (BPI), located at the municipality of Botucatu, state of São Paulo, Brazil. The resulting sequences were confirmed by the analysis of both strands and the consensus sequence was obtained using the Geneioys 2021.1.1 program (Kearse et al., 2012). The final sequences were then trimmed to start and end for alignment, and pseudogenes presence was checked. All the obtained sequences were deposited at GenBank database (https://www.ncbi.nlm.nih.gov/genbank/).

The sequences were compared with the ones from the database sequence collection by the means of the Basic Local Alignment Search Tool (BLAST), as implemented on the NCBI website. To verify the identification, we added each sequence using the nblast section of BLAST e searched for similar sequences present at the database. After the search, we assured that the results were organized by the nearest identity.

We used the IQ-TREE program for the maximum likelihood phylogenetic analysis for each gene (Miller et al., 2010), with Cytochrome c oxidase gene subunit 1 (COI). The evolutionary model that better fit the data was determined by the IQ-TREE following the Bayesian Information Criterion (BIC) (Luo et al., 2010), and used to infer the tree. Branch support was validated by ultrafast bootstrap with 1000 replica.

Results

Taxonomy

Order Amphipoda Latreille, 1816

Suborder Senticaudata Lowry & Myers, 2013

Family Hyalellidae Bulycheva, 1957

Genus *Hyalella* Smith, 1874

Hyalella sp1 n. sp. Penoni & Bueno (Figs. 2–6)

Type material: Holotype male (Fig. 2A), body length = 7.25 mm, head length = 0.6 mm, "Gruta da Água Sumida" Cave, Parque Estadual Turístico do Alto Ribeira, Iporanga municipality, São Paulo state, Brazil (24° 28' 42" S; 48° 37' 44" W), MNRJcarcino XXXXX, July 2018, Bichuette, M.E., Zepon, T. coll; allotype female (Fig. 3B), body length = 3.87 mm, head length = 0.4 mm, MNRJcarcino XXXX, July 2017, Bichuette, M.E., Zepon, T., coll.; paratype one male, CCUFLA 455, Nov. 2009, Bichuette, M.E., Zepon, T., two males, LES0018586 and LES0018591, Apr. 2018, Bichuette, M.E., Zepon, T. cols., two males in slides CCUFLA 456 (same collection data as holotype).

Diagnosis: Body surface smooth. Epimeron 2 postero-distal margin acute. Antenna 1 and 2 shorter than half the body length. Maxilla 1 palp longer than half the distance between the base of the palp and the base of setae on outer plate; outer plate with 9 serrate setae. Gnathopod 1 carpus with serrate seta on the anterior margin. Gnathopod 2 propodus ovate, palm subequal to posterior margin of propodus and with a minute posterior excavation, slope oblique. Uropod 1 of the male without a curved seta on inner ramus. Uropod 3 longer than the telson, peduncle and ramus subequal. Coxal gills on segments 2–6. Sternal gills on segments 2–7.

Male: Mean body length 8.93 ± 2.2 mm (N = 3) (7.25 – 11.45 mm); mean head length 0.9 ± 0.25 mm (N = 3) (0.6 – 0.87 mm). Body surface smooth. Epimeral plates 2 accuminated (Fig. 3I). Coxae 1–3 similar, subequal in size; coxa 4 as wide as long, excavated posteriorly; coxae 5–7 with some scattered microtrichs. Eyes present.

Antenna 1 (Fig. 3A) shorter than half the body length (0.3× the body length), 0.8× the length of Antenna 2, 1.6× longer than the peduncle of Antenna 2 and 1.5× longer than the flagellum of Antenna 2. Peduncle articles 1–3 relative length 1:0.82:0.63; flagellum with 17–18 articles; aesthetascs occurring distally on flagellum starting at article 5 until the last one.

Antenna 2 (Fig. 3B) not reaching half the body length (0.4× the body length); peduncle slender, almost 4× the length of the head; flagellum with 15 articles, 1.1× longer than the peduncle.

Mandible without palp; incisor toothed; left (Fig. 3C) *lacinia mobilis* with 5 teeth, setae row with 4 pappose setae, an accessory seta on the molar process; right mandible (Fig. 3D) with 2 pappose setae and some setules; molar process cylindrical with an accessory seta.

Upper lip (Fig. 3E) margin rounded; distal border covered by setules. Lower lip (Fig. 3F) outer lobes rounded, distally notched, covered by setules apically.

Maxilla 1 (Fig. 3G) inner plate slender, with 2 apical pappose setae and lateral setules. Outer plate with 9 serrate setae, and a few setules on the inner margin, apically. Palp relatively long, uniarticulate, longer than wide, reaching more than half the distance between base of the palp and base of the setae on the outer plate, with 1 simple seta.

Maxilla 2 (Fig. 3H) inner plate shorter than outer plate, inner plate with 2 strong pappose setae distally and some simple and pappose setae disto-apically, and setules laterally; outer plate with long simple setae and some papposerrate setae, disto-apically, and setules laterally.

Maxilliped (Fig. 3J) without comb scales; inner plate with 3 cuspidate distal setae apically, a few pappose setae disto-apically; outer plate with several long simple setae on the inner margin and 2 smaller simple setae on the outer margin; palp longer than inner and outer plates, with 4 articles; article 1 inner margin with up to 4 long simple setae and outer margin with 3 simple setae; article 2 inner margin with several long simple setae and outer margin with 4 long simple setae; article 3 distal inner and outer margins with several long and thin simple setae; article 4 (dactylus) unguiform with distal nail present, with up to 5 simple setae, shorter than nail.

Gnathopod 1 (Fig. 4A) subchelate; coxal plate 1.7× wider than long, with simple setae on the margin and up to 3 serrate setae on the posterior inner face, close to the attachment of the basis; basis with 5 serrate setae on the posterior margin, 1 small simple setae on the disto-anterior margin and 2 serrate setae on the disto-posterior margin; ischium with 1 serrate and 1 simple setae postero-apically; merus with 7 serrate setae and 1 pappose seta posteriorly; carpus longer than wide, with pappose setae on the lateral distal lobe, 5 pappose setae on inner margin, up to 9 serrate setae antero-distally, and 2 serrate setae on the anterior margin; propodus 1.5× longer than wide, hammer shaped, up to 10 long simple setae on disto-posterior margin, comb-scales on the disto-posterior corner, numerous long and short simple setae on disto-anterior margin; palm slope transverse, with several long and short simple setae; up to 10 pappose setae and 1 cuspidate seta with accessory seta on inner face; dactylus claw-like, with polygonal pattern and a plumose seta distally.

Gnathopod 2 (Fig. 4B) subchelate, without comb scales; coxal plate 1.9× wider than long, with simple setae on the margin; basis with 6 long serrate setae on posterior margin, 1 short simple setae on anterior margin, and up to 2 serrate setae disto-posteriorly; ischium with 3 serrate setae disto-posteriorly; merus with 9 serrate setae, divided in 2 groups, disto-posteriorly; carpus lobe slim, produced between merus and propodus, posterior margin with pappose setae, disto-anterior margin with 5 serrate setae; propodus ovate, posterior margin 1.3× longer than the palm, slope oblique, posterior margin with several short simple setae, disto-posterior corner with polygonal pattern, palm posteriorly excavated, some short cuspidate setae with accessory setae and some simple setae, anterior margin with a few simple setae; dactylus claw-like, congruent with palm and with one plumose seta distally.

Pereopods 3–7 (Fig. 4C–G) simple. Simple setae distally on all coxae, and a few microtrichs on inner face of coxae 5–7; coxa 3 similar to 1 and 2; coxa 4 as long as wide, excavated posteriorly; coxa 5 posterior lobe longer than anterior lobe; coxa 6 anterior lobe reduced; coxa 7 reduced. Pereopods 3–5 subequal in size, pereopod 6 1.3× longer than P3–5 and slightly longer than P7. Pereopods 3 and 4 basis with 2 to 3 groups of 1 or 2 serrate setae on the posterior margin, 2 to 3 serrate setae on the disto-posterior margins of basis and ischium, 3 to 5 groups of 1 to 3 simple setae on the posterior margin of the merus, 4 to 7 groups of simples and/or cuspidate setae with accessory setae on the posterior margins of carpus and propodus; pereopod 3 merus with 2 cuspidate seta with accessory seta on the anterior margin. Pereopods 5–7 with small simple setae on the posterior margin of the basis, 2 or 3 serrate setae postero-apically, 7 to 9 groups of 1 to 2 cuspidate setae with or without accessory seta on the anterior margins of the basis, and up to 5 cuspidate setae with accessory seta on the disto-anterior margin; merus posterior margins with 2 curved cuspidate setae with accessory seta; merus, carpus and propodus anterior margins with 2 to 5 groups of cuspidate setae with or without accessory seta and simple setae, disto-anterior and disto-posterior margins with up to 9 cuspidate setae with accessory seta. Pereopods 3–7 dactylus with a plumose seta.

Pleopods (Fig. 5A) peduncle half the length of the rami, no coupling spines observed; both rami with several long plumose setae.

Uropod 1 (Fig. 5B) peduncle 1.3× longer than inner ramus and 1.5× longer than the outer ramus, with up to 6 cuspidate setae with accessory seta; inner ramus 1.2× longer than the outer ramus, with 3 cuspidate setae with accessory seta on dorsal margin and 5 cuspidate setae (3 longer, 2 shorter) apically; outer ramus with 3 cuspidate setae on dorsal margin and 4 cuspidate setae (2 longer, 2 shorter) apically.

Uropod 2 (Fig. 5C) less than 0.7× the total length of Uropod 1, 1.2× longer than the peduncle of Uropod 1, peduncle subequal to inner ramus, 1.2× longer than the outer ramus, with 3 cuspidate setae; inner ramus 1.2× longer than outer ramus, with 2 cuspidate setae with accessory seta on dorsal margin and 4 cuspidate setae (2 longer, 2 shorter) apically; outer ramus with 3 cuspidate setae with accessory seta on dorsal margin and 4 cuspidate setae (2 longer, 2 shorter) apically.

Uropod 3 (Fig. 5D) half the length of the peduncle of uropod 1 and 0.9× the length of the peduncle of uropod 2, 1.2× longer than the telson; peduncle with 4 cuspidate setae on inner distal margin; inner ramus absent; outer ramus with a small dentation midway bearing 1 cuspidate seta with accessory seta, and 5 cuspidate setae with an accessory seta and a triangular cuspidate seta apically.

Telson (Fig. 5E) entire, as long as wide, apically rounded with 2 simple setae; 2 small plumose setae on each side, symmetrically.

Epimeron (Fig. 3I) 1 and 3 postero-distal margin sub-acute, epimeron 2 postero-distal margin acute; first epimeron shorter than the second and third.

Coxal gills sac-like on pereonites 2-6. Sternal gills tubular on pereonites 2-7.

Female: (Fig. 2B). Allotype female not dissected. Body length = 3.87 mm; head length = 0.4 mm.

Habitat: Freshwater, hypogean, troglophile. A few specimens of *Hyalella sp1* n. sp. were found on the water pools of Gruta da Água Sumida Cave.

Conservation: H. sp1 n. sp. is classified as endangered (EN) based on IUCN subcategory B1 (< 5.000 km^2 in area of occurrence extension) and D1 (< 250 mature individuals) (ICMBIO, 2013).

Remarks: Hyalella sp1 n. sp. is troglophile species, with no apparent adaptations to the life underground. One unusual characteristic is the presence of setae on the anterior margin of the carpus of gnathopod 1 (besides the ones present at the disto-anterior margin), which differentiate it from almost all of the known species of the genus. For the Brazilian species, *H. sp1* n. sp. is different from 21 described species of *Hyalella* by the absence of a curved seta on the inner ramus of the male uropod 1. There are other 19 species (described or under description) that lack this seta. We can distinguish *H. sp1* n. sp. from *H. caeca* Pereira, 1989, *H. sp2* and *H. troglofugia* Bastos-Pereira, Oliveira & Ferreira, 2018, because these are species that lack eyes and have the maxilla 1 palp short. The relative size of the palp can actually be used to differentiate the new species from all but one (*H. temimina*) of these 19 species. One important difference between the new species and *H. temimina* is the comb scale and polygonal pattern presence and distribution at the gnathopods. And the new species has an Antenna 2 shorter than half the body length, which is different from *H. spelaea* Bueno & Cardoso, 2011 (Cardoso et al., 2011), *H. sp3*, *H. gracilicornis* (Faxon, 1876) (González & Watling, 2003), *H. longiprodus* Limberger, Graichen & Castiglioni, 2021 (Limberger et al., 2021), *H. longistila* (Faxon, 1876) (González & Watling, 2003) and *H. warmingi* Stebbing, 1899 (González & Watling, 2003) that have Antenna 2 longer than half the body length, and from *H. sp4*, *H. sp5* Penoni & Bueno (in press) (Penoni et al., in press – B), *H. virgineae* that have Antenna 2 as long as half the body length.

Reconstructed phylogeny

Two proposed trees were generated, one for the 16S gene and another for the 18S gene (Figs. 6 and 7). It was not possible to recover good sequences of both genes for all the analyzed species, so that the two trees were generated based on different species. Here we observe the grouping of geographically close species, with *Hyalella* sp6. (from Nobres/MT) and *H. veredae* (from Presidente Olegário/MG) consistently separated on exclusive groups apart from the species from Apiaí, Eldorado, Iporanga, São Luís do Paraitinga and Ribeirão Grande (state of São Paulo).

On the first tree (Fig. 6), the branch formation sustains that *Hyalella* populations from different collection sites are molecularly different from one another. It indicates that the understanding that these are different species is a valid hypothesis. Two branches should be more closely observed: the *Hyalella temimina – Hyalella sp3*; and the *Hyalella bocaina – Hyalella* sp7. The first pair is composed of two species from caves located at the municipality of Iporanga (Temimina II cave and Santana cave – both from PETAR), but morphological analysis indicate consistent differences. The second pair is formed by species from Parque Estadual Intervales, and the morphology should be better analyzed to reach any conclusions. *Hyalella sp1* n. sp. branches out from other species of the same geographical region and is sister species with the ones from Santana cave (epigean), Temimina II cave, Gruta do Fendão cave and from Rio Bocaina (Parque Estadual Intervales).

On the second tree (Fig. 7), we also see a consistent branch division. The two populations of *H. veredae* are molecularly very similar, and previous morphological observations didn't indicate any major differences, which suggests that this species is present in more than one cave, with multiple populations. Here we see the separation of *H. sp2* and *H. sp3* in two separated terminal groups. *Hyalella sp2* is grouped with *H. bocaina* and *H. sp3* is grouped with *H. sp4*, and not with one another.

Finally, the reconstructed molecular phylogeny of these *Hyalella* species resulted in no major differences between the specimens of *H. sp4* found both inside and outside of the Gruta da Tapagem Cave (Caverna do Diabo). Together with the morphological analysis (personal observation), it suggests that they are all one single troglophile species, capable of sustaining health epigean and hypogean populations.

Final remarks

The morphology of *Hyalella* is well conserved among different species (González & Watling, 2002; Bueno et al., 2014), and a thorough examination of morphological details is essential for species identification and description. There is no single structure or morphological detail that can be used to unequivocally identify a species in this genus. Hence, the use of molecular tools can enlighten the understanding of species boundaries, especially when dealing with geographically close species with similar morphologies.

We produced two reconstructed phylogenetic trees, each one based on a different DNA gene. Not all species studied here could be placed on both reconstructed phylogenetic trees. Yet, we could see that even species like the *sp2-sp3* duo, which are only meters apart from each other (personal observation), were separated in at least one of the trees.

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References

Bastos-Pereira, R., Oliveira, M. P. A. & Ferreira, R. L. (2018). Anophtalmic and epigean? Description of an intriguing new species of *Hyalella* (Amphipoda, Hyalellidae) from Brazil. *Zootaxa*, *4407*(2): 254–266. https://doi.org/10.11646/zootaxa.4407.2.6

Bueno, A. A. P., Rodrigues, S. G. & Araujo, P. B. (2014). O estado da arte do gênero *Hyalella* Smith, 1874 (Crustacea, Amphipoda, Senticaudata, Hyalellidae) no Brasil. In: *HAYASHI, C. (Ed.). Tópicos de Atualização em Ciências Aquáticas*. Uberaba: UFMT, 2014. p. 57-88.

Bulycheva, A. (1957) The sea fleas of the USSR and adjacent waters (Amphipoda: Talitroidea). Keys to the Fauna of the USSR. *Akademiia Nauk SSSR, Opredeliteli po Faune SSSR, 65*, 1–185 (in Russian).

Cardoso, G. M., Bueno, A. A. P. & Ferreira, R. L. (2011). A new troglobiotic species of *Hyalella* (Crustacea, Amphipoda, Dogielinotidae) from Southeastern Brazil. *Nauplius, 19*(1): 17–26.

Deotti, C. M., Penoni, L. R., Bichuette, M. E. & Bueno, A. A. P. (in press). Expanding the knowledge of *Hyalella* Smith, 1874 (Amphipoda, Hyalellidae) for southeast Brazil: two new troglophilic species from caves of the Atlantic Rainforest. *Nauplius*.

Estoup, A.; Lagiardèr, C.R.; Perrot, E. & Chourrout, D. 1996. Rapid one-tube DNA extraction for reliable PCR detection of fish polypmorphic markers and transgenes. *Molecular Marine Biology and Biotechnology*, 5: 295–298.

Gonzalez, E. R. & Watling, L. (2002). Redescription of *Hyalella azteca* from its type locality, Vera Cruz, México (Amphipoda: Hyalellidae). *Journal of Crustacean Biology, 23*(1): 173–183. doi:10.1163/20021975- 99990220

González, E. R., & Watling, L. (2003). A new species of *Hyalella* from Brazil (Crustacea: Amphipoda: Hyalellidae), with redescriptions of three other species in the genus. *Journal of Natural History, 37*(17), 2045-2076.

González, E. R. (2003). The freshwater amphipods *Hyalella* Smith, 1874 in Chile (Crustacea: Amphipoda). *Revista Chilena de Historia Natural*, *76*(4), 623-637.

Kearse, M.; Moir, R.; Wilson, A.; Stones-Havas, S.; Cheung, M.; Sturrock S; Buxton, S.; Cooper, A.; Markowitz, S.; Duran, C.; Thierer, T.; Ashton, B.; Meintjes, P.; &

Drummond, A. 2012. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics*, 28(12): 1647–1649. https://doi.org/10.1093/bioinformatics/bts199

Jurado-Rivera, J. A., Pons, J., Alvarez, F., Botello, A., Humphreys, W. F., Page, T. J., ... & Jaume, D. (2017). Phylogenetic evidence that both ancient vicariance and dispersal have contributed to the biogeographic patterns of anchialine cave shrimps. *Scientific Reports*, *7*(1), 1-11.

Jurado-Rivera, J. A., Zapelloni, F., Pons, J., Juan, C., & Jaume, D. (2020). Morphological and molecular species boundaries in the *Hyalella* species flock of Lake Titicaca (Crustacea: Amphipoda). *Contributions to Zoology*, *89*(4), 353-372.

Latreille, P.A. (1802). *Histoire Naturelle, générale et particulière des Crustacés et des Insectes*. Vol. 1–4. Paris.

Limberger, M., Castiglioni, D. S. & Graichen, D. A. S. (2021). A new species of freshwater amphipod (Crustacea, Peracarida, Hyalellidae) from Southern Brazil. *Zootaxa, 5026*(2): 182 –200. https://doi.org/10.11646/zootaxa.5026.2.2

Limberger, M., Santos, S. & Castiglioni, D. D. S. (2022). *Hyalella luciae* (Crustacea, Amphipoda, Hyalellidae) – a new species of freshwater amphipod from Southern Brazil. *Zootaxa*, 5174(5), 568-582.

Lowry, J. K., & Myers, A. A. (2013). A phylogeny and classification of the Senticaudata subord. nov. Crustacea: Amphipoda. *Zootaxa, 3610(1)*, 1-80.

Lukhtanov, V. A. (2019). Species delimitation and analysis of cryptic species diversity in the XXI century. *Entomological Review, 99(4)*, 463-472.

Luo, A.; Qiao, H.; Zhang, Y.; Shi, W.; Ho, S.Y.; Xu, W.; Zhang, A. & Zhu, C. 2010. Performance of criteria for selecting evolutionary models in phylogenetics: a comprehensive study based on simulated datasets. BMC *Evolutionary Biology*, 10(242): 1–13. https://doi.org/10.1186/1471-2148-10-242

Medlin, L.; Elwood, H.J.; Stickel, S. & Sogin, M.L. 1988. The characterization of enzymatically amplified eukaryotic 16S-like rRNA-coding regions. *Gene*, 71: 491–499. https://doi.org/10.1016/0378-1119(88)90066-2

Miller, M.A.; Pfeiffer, W. & Schwartz, T. (2010). Creating the CIPRES Science Gateway for inference of large phylogenetic trees. Gateway Computing Environments Workshop (GCE). https://doi.org/10.1109/GCE.2010.5676129

Penoni, L. R., Lares, M. M. A. & Bueno, A. A. P. (2021). Description of a new species of the freshwater amphipod *Hyalella* Smith, 1874 (Amphipoda: Hyalellidae) from southeastern Brazil, with remarks on their population biology and reproduction. *Journal of Crustacean Biology, 41(3)*, ruab050. https://doi.org/10.1093/jcbiol/ruab050

Penoni, L. R., Bichuette, M. E. & Bueno, A. A. P. (in press – A). Two new species of *Hyalella* Smith, 1874 (Crustacea: Amphipoda: Hyalellidae, and redescription of *Hyalella caeca* Pereira, 1989.

Penoni, L. R., Bichuette, M. E. & Bueno, A. A. P. (in press – B). Two new species of *Hyalella* Smith, 1874 (Amphipoda: Hyalellidae) from Southeast Brazil, with a pictorial key to the hypogean species of the genus. *Zootaxa*.

Pereira, V. F. G. C. (1989). Uma nova espécie de anfípode cavernícola do Brasil: *Hyalella caeca* n. sp. (Amphipoda, Hyalellidae). *Revista Brasileira de Zoologia*, 6, 49-55.

Rangel, C., Silva, A. L. L., Siegloch, A. E., Limberger, M. & Castiglioni, D. D. S. (2022). First island species of *Hyalella* (Amphipoda, Hyalellidae) from Florianpolis, state of Santa Catarina, Southern Brazil. *Zootaxa, 5116(1)*, 40-60.

Rodrigues, S. G. 2016. *Filogenia molecular, biogeografia e estrutura populacional de anfípodos de água doce (Crustacea, Hyalellidae) da América do Sul.* Tese de Doutorado. Universidade Federal de Lavras, Lavras, Brazil. 125p.

Rogers, D.C., Magalhães, C., Peralta, M., Ribeiro, F.B., Bond-Buckup, G., Price, W.W., Guerrero-Kommritz, J., Mantelatto, F.L., Bueno, A., Camacho, A.I., González, E.R., Jara, C.G., Pedraza, M., Pedraza- Lara, C., Latorre, E.R., & Santos, S. (2020). Phylum Arthropoda: Crustacea: Malacostraca. In: *Thorp and Covich's freshwater invertebrates* (J.H. Thorp & D.C. Rogers, eds), pp. 809–986. Academic Press, London.

Sambrook, J.; Fritsch, E. R.; & Maniatis, T. 1989. *Molecular Cloning: A Laboratory Manual* (2nd ed.). Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Smith, S.I. 1874. The Crustacea of the freshwaters of the United States. A synopsis of the higher freshwater Crustacea of the Northern United States, Appendix F. Natural History. *United States Commission of Fish and Fisheries, 2*, 637–661.

Zapelloni, F., Pons, J., Jurado-Rivera, J. A., Jaume, D. & Juan, C. (2021). Phylogenomics of the *Hyalella* amphipod species-flock of the Andean Altiplano. *Scientific reports, 11*(1), 1-14.

Zimmer, A., Araujo, P. B. & Bond-Buckup, G. (2009). Diversity and arrangement of the cuticular structures of Hyalella (Crustacea: Amphipoda: Dogielinotidae) and their use in taxonomy. *Zoologia (Curitiba), 26,* 127-142.

Figures:

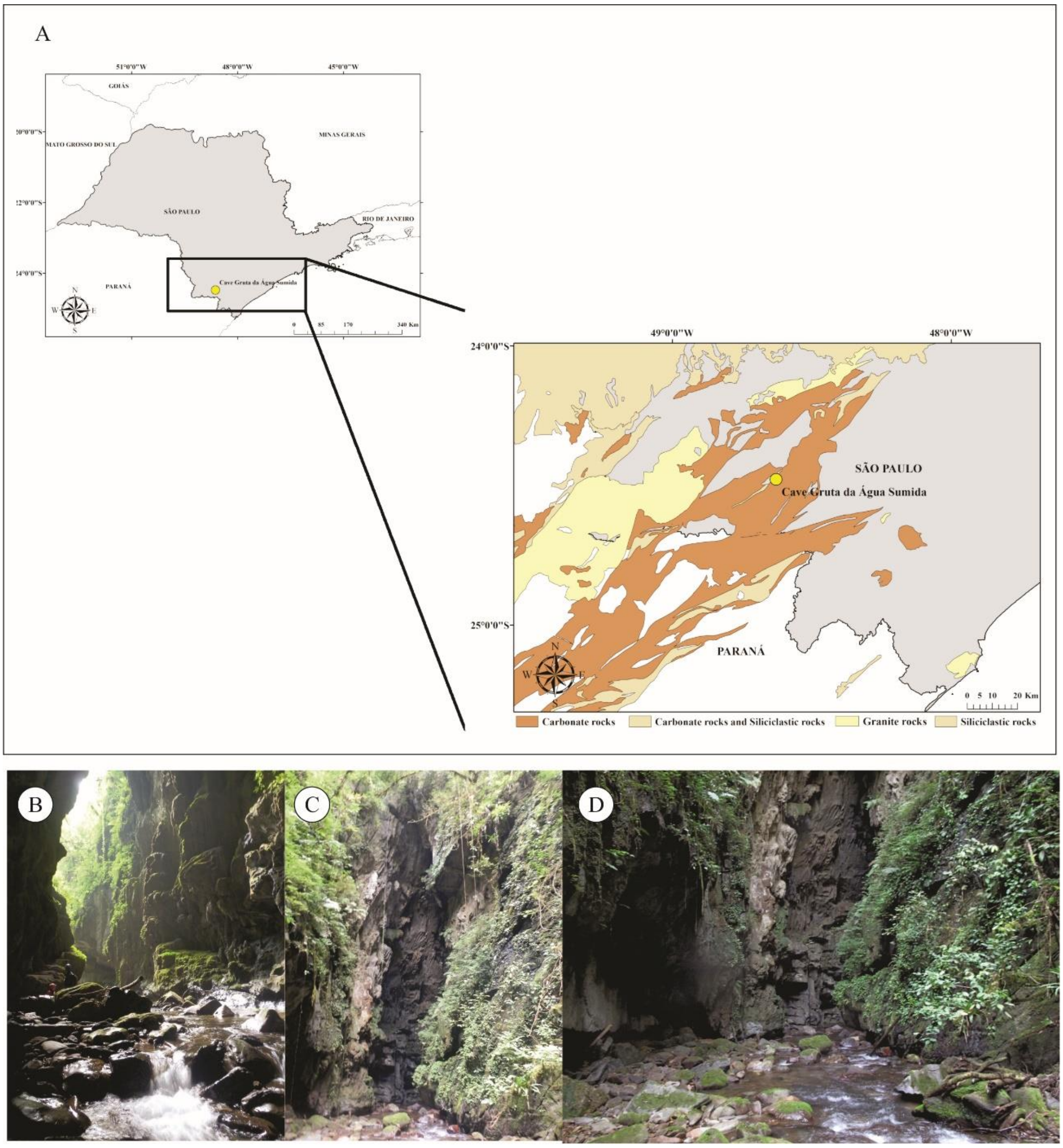

Figure 1: Collection site of *Hyalella sp1* n. sp. – Gruta da Água Sumida Cave, Parque Estadual Turístico do Alto Ribeira (24° 28’ 42” S; 48° 37’ 44” W), municipality of Iporanga, state of São Paulo. Map of the karst region of Gruta da Água Sumida Cave, with highlights for the cave location (**A**); Hypogean entrance zone of Gruta da Água Cave Sumida (**B**); Epigean portion of cave entrance of Gruta da Água Sumida Cave (**C** and **D**). Photographs: von Shimonsky, D. (**A** and **B**); Zepon, T. (**C**).

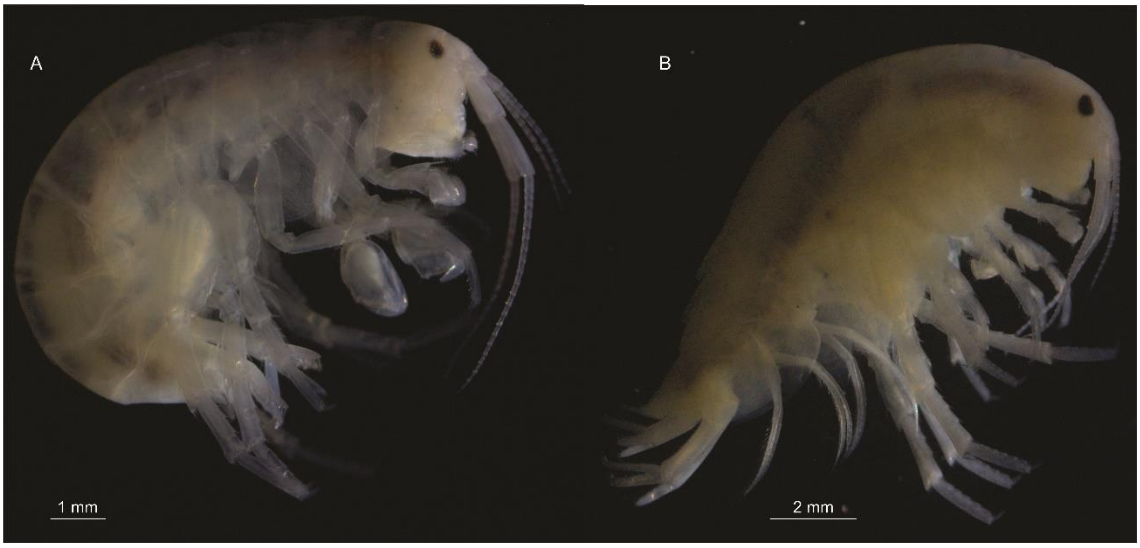

Figure 2: *Hyalella sp1* n. sp., Gruta da Água Sumida Cave, Parque Estadual Turístico do Alto Ribeira (24° 28’ 42” S; 48° 37’ 44” W), municipality of Iporanga, state of São Paulo. Holotype male, x.x mm (MNRJcarcino xxxxx) (**A**). Allotype female, x.x mm (MNRJcarcino xxxxx) (**B**). Scale bars: A = 1 mm; B = 2 mm.

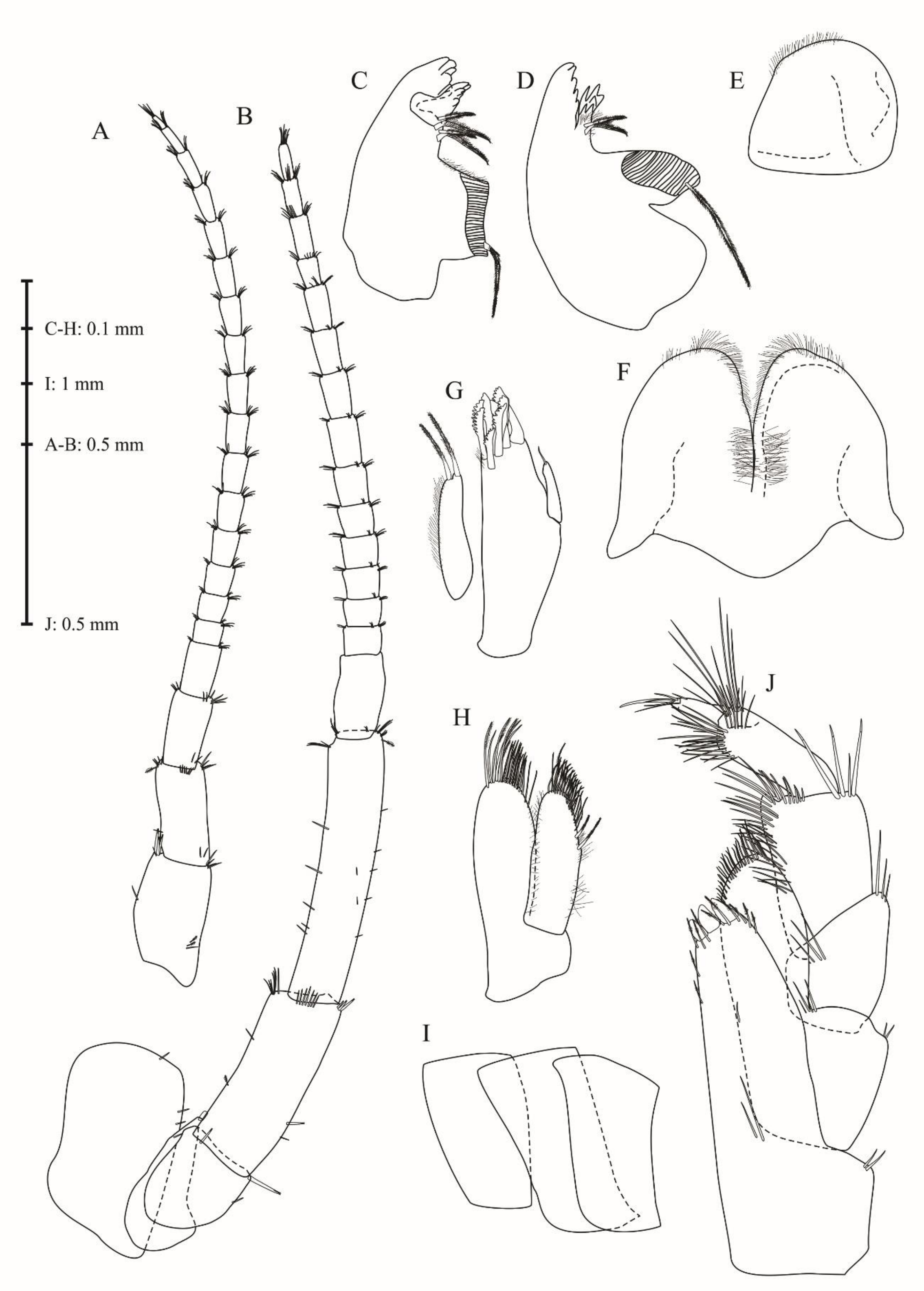

Figure 3: *Hyalella sp1* n. sp., Gruta da Água Sumida Cave, Parque Estadual Turístico do Alto Ribeira (24° 28’ 42” S; 48° 37’ 44” W), municipality of Iporanga, state of São Paulo. Paratype male, 8.09 mm; antenna 1 (**A**); antenna 2 (**B**); left mandible (**C**); right mandible (**D**); upper lip (**E**); lower lip (**F**); maxilla 1 (**G**); maxilla 2 (**H**); epimeral plates (**I**); maxilliped (**J**). Scale bars: A and B = 0.5 mm; C–H = 0.1 mm; I = 1 mm; J = 0.5 mm.

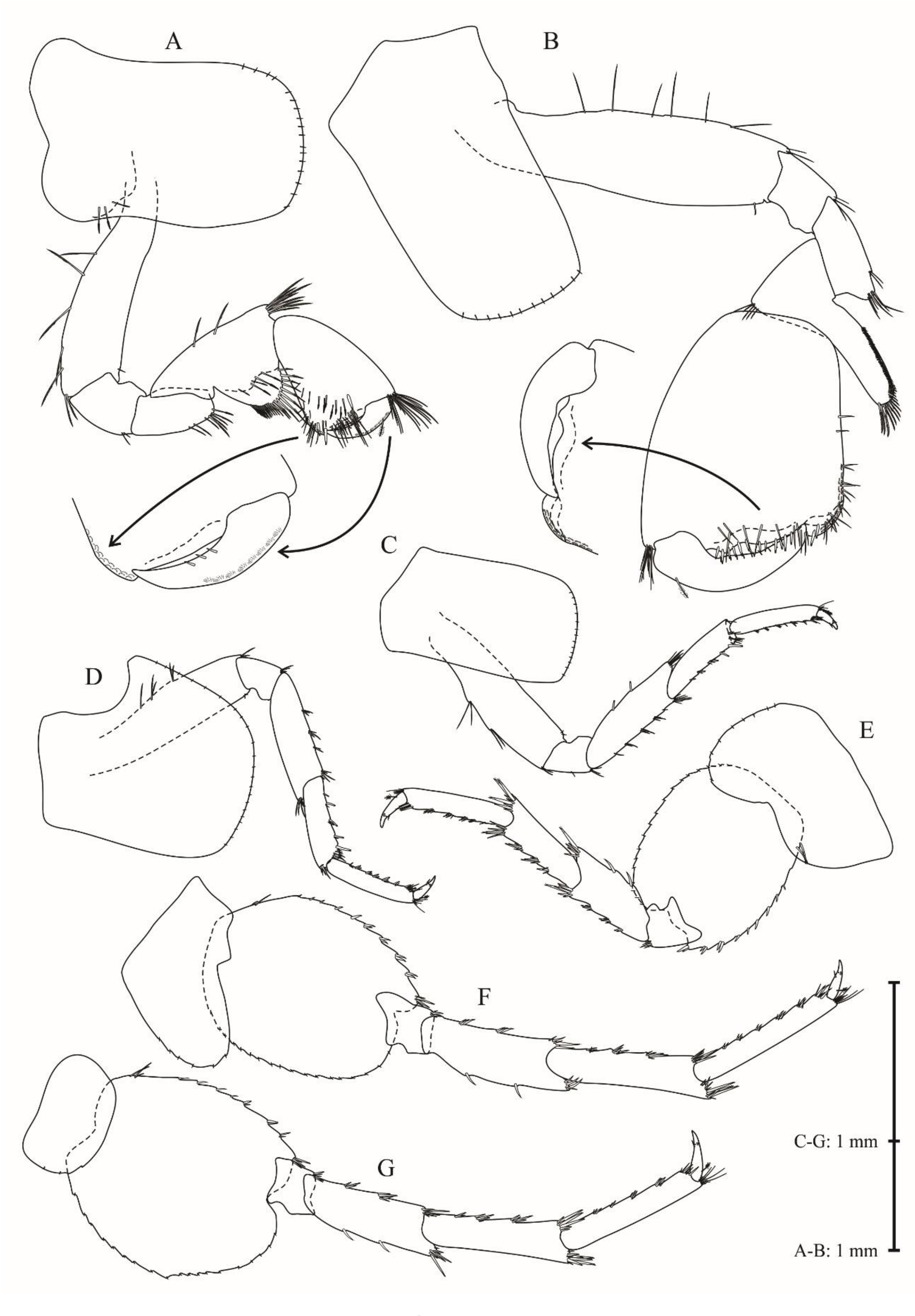

Figure 4: *Hyalella sp1* n. sp., Gruta da Água Sumida Cave, Parque Estadual Turístico do Alto Ribeira (24° 28’ 42” S; 48° 37’ 44” W), municipality of Iporanga, state of São Paulo. Paratype male, 8.09 mm; gnathopod 1 (**A**); gnathopod 2 (**B**); pereopod 3 (**C**); pereopod 4 (**D**); pereopod 5 (**E**); pereopod 6 (**F**); pereopod 7 (**G**). Scale bars = 1 mm.

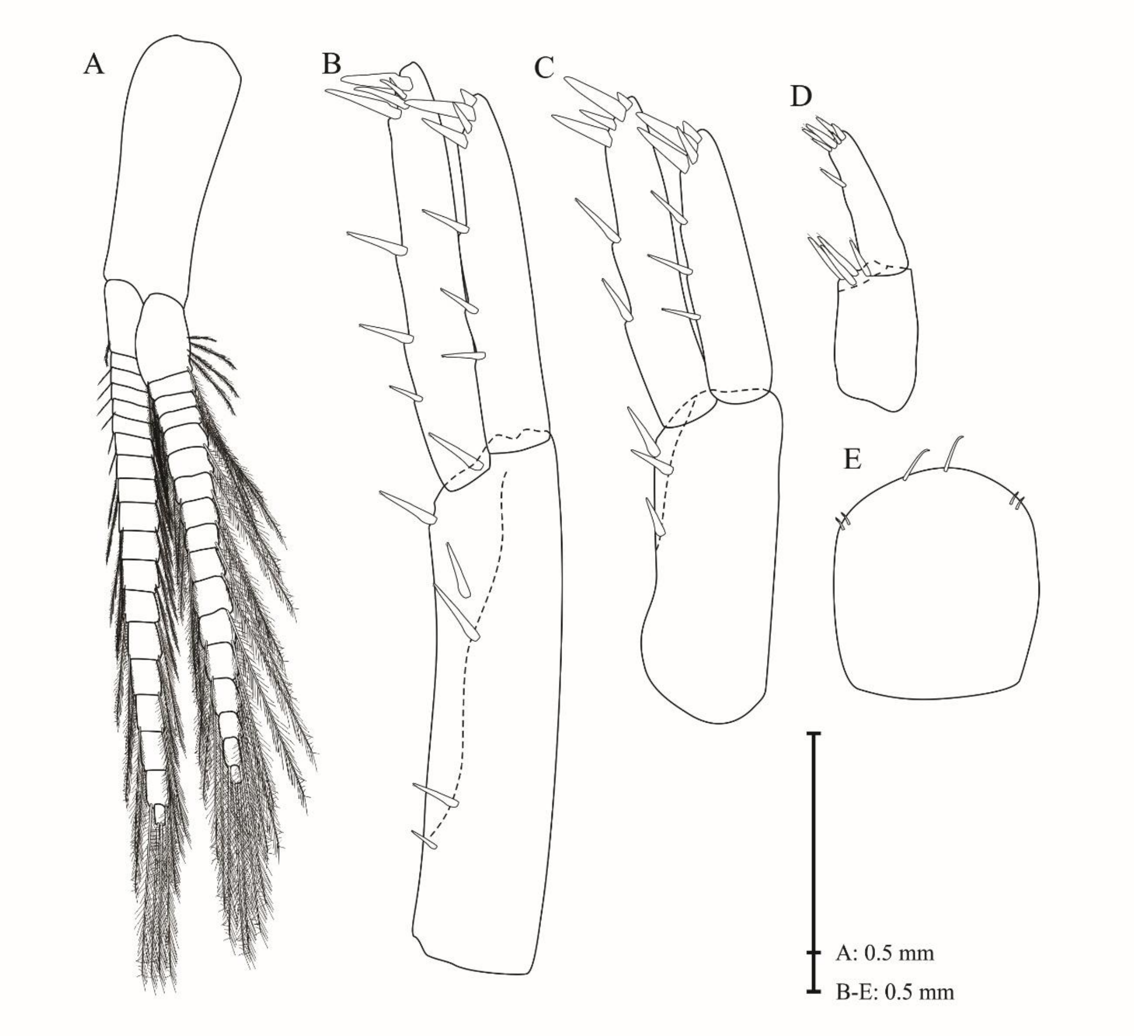

Figure 5: *Hyalella sp1* n. sp., Gruta da Água Sumida Cave, Parque Estadual Turístico do Alto Ribeira (24° 28’ 42” S; 48° 37’ 44” W), municipality of Iporanga, state of São Paulo. Paratype male, 8.09 mm; pleopod (**A**); uropod 1 (**B**); uropod 2 (**C**); uropod 3 (**D**); telson (**E**). Scale bars = 0.5 mm.

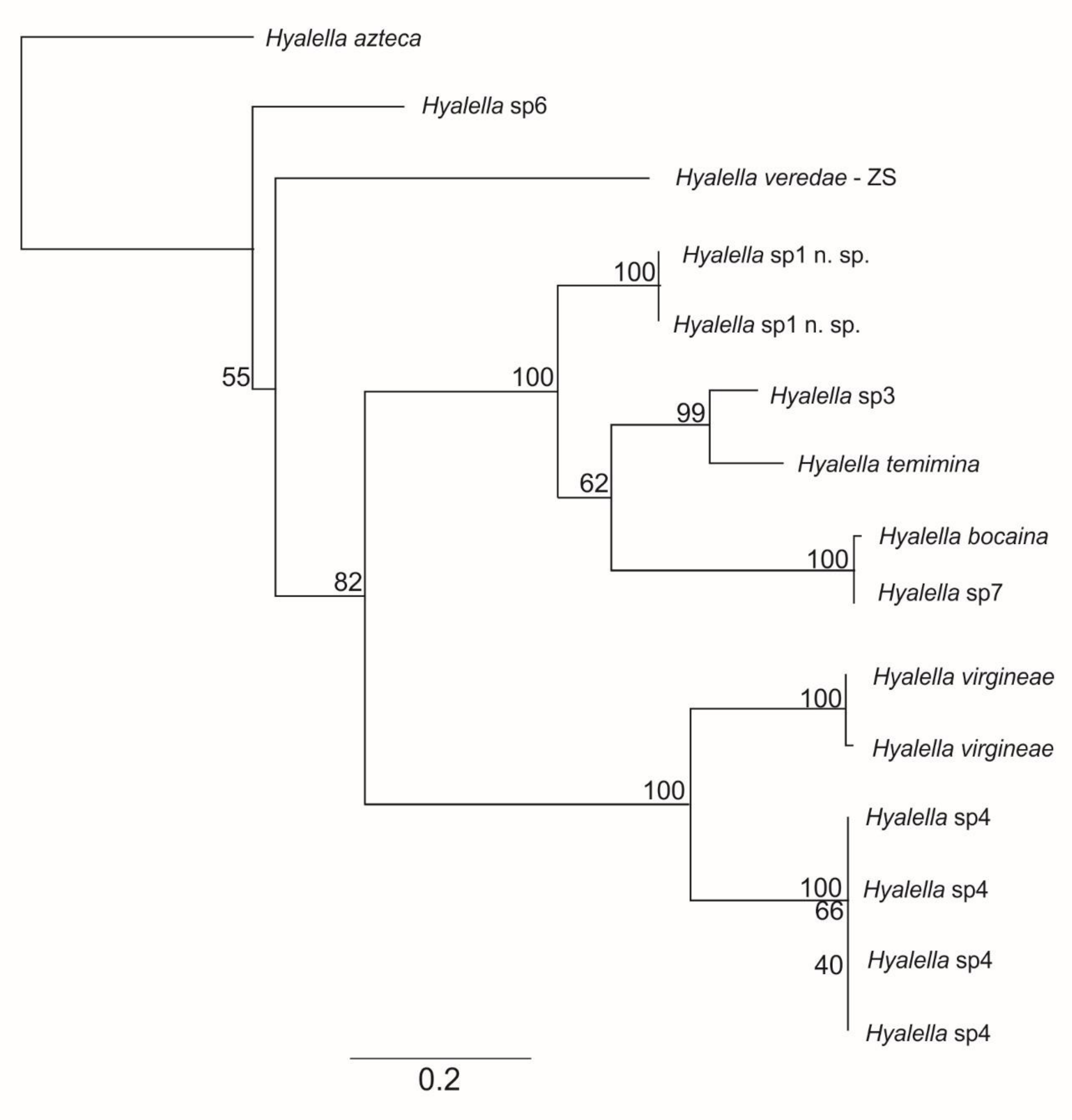

Figure 6: Reconstructed phylogeny of cave-related *Hyalella* Smith, 1874 from Southeast Brazil based on the mitochondrial 16S gene. Numbers at nodes are bootstrap values. *Hyalella azteca* was used as an outgroup.

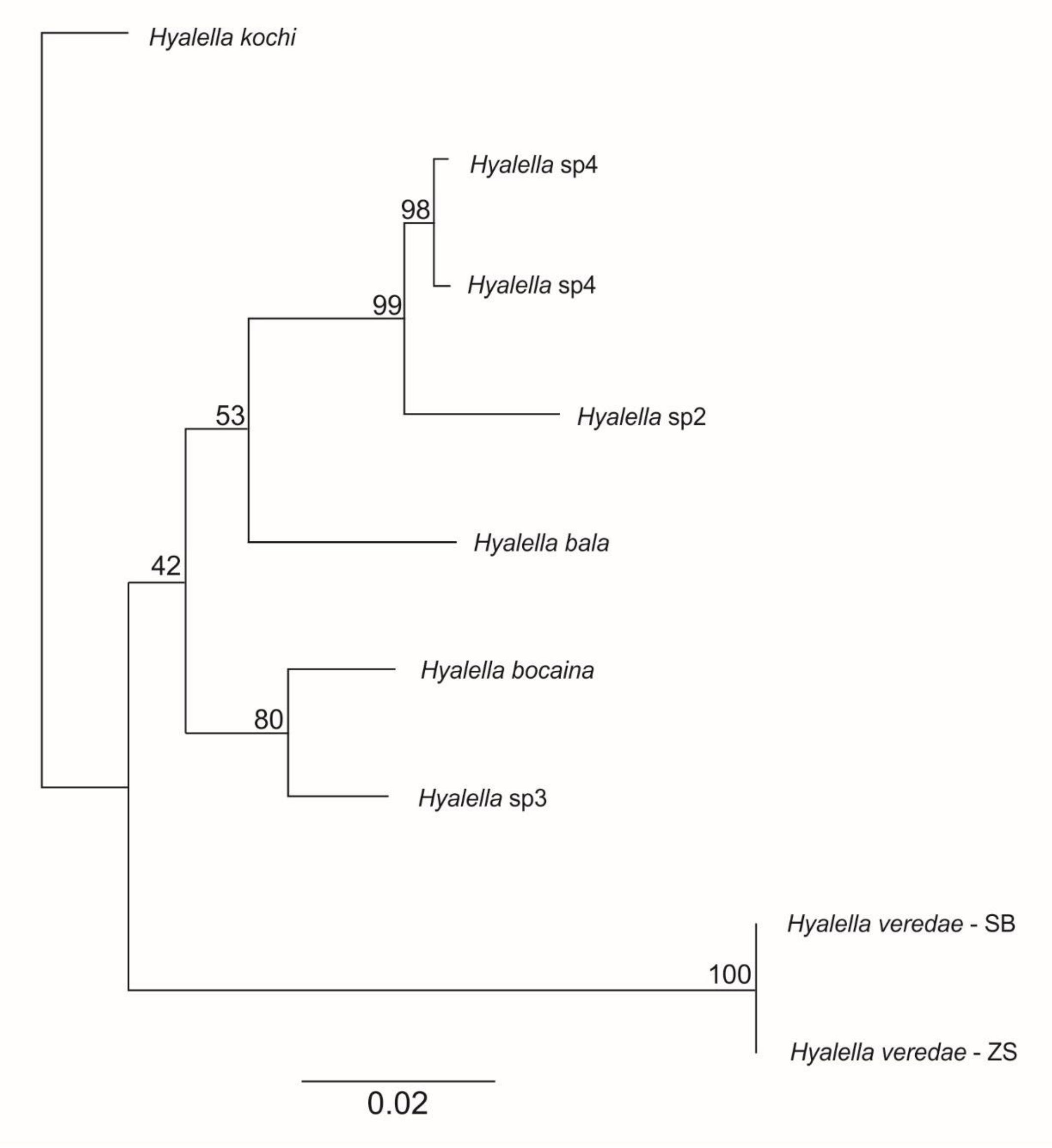

Figure 7: Reconstructed phylogeny of cave-related *Hyalella* Smith, 1874 from Southeast Brazil based on the nuclear 18S gene. Numbers at nodes are bootstrap values. *Hyalella kochi* was used as an outgroup.

3. Capítulo 3

Artigo: Two new species of *Hyalella* Smith, 1874 (Crustacea: Amphipoda: Hyalellidae), and redescription of *Hyalella caeca* Pereira, 1989

Artigo submetido para o periódico Zootaxa

Two new species of *Hyalella* Smith, 1874 (Crustacea: Amphipoda: Hyalellidae), and redescription of *Hyalella caeca* Pereira, 1989

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Abstract

We describe two new species of the genus *Hyalella* found at Parque Estadual Turístico do Alto Ribeira - PETAR, a state park in Southeastern Brazil and redescribe *H. caeca*, the first cave species of the genus described to Brazil. *H. caeca* is found at Tobias de Baixo Cave and its original description lacks some important morphological features; the animals are completely blind and depigmented, with antenna 2 almost half the body length, maxilla 1 with 9 serrate setae on the outer plate, gnathopod 1 with polygonal patter on the dactylus, gnathopod 2 with a slender posterior excavation and uropod 3 subequal in length to the peduncle of uropod 2 and the telson. The newly described species are found one inside of Santana cave and the other outside of the same cave, but their morphologies differ greatly. *H. sp2* n. sp. is also blind and depigmented, with elongated antennas and legs; its main features include antenna 1 more than half the body length, maxilla 1 with 8 serrate setae on the outer plate, gnathopod 1 of male and female identical, with comb scales on the merus, and uropod 3 longer than the telson. The other new species, *H. sp3* n. sp. has normal eyes and pigmentation, and its main features include antenna 1 half the size of antenna 2, maxilla 1 outer plate with 9 serrate setae, gnathopod 1 propodus with polygonal pattern, gnathopod 2 palm with slender posterior excavation, and uropod 3 ramus with an excavation midway to the apex.

Key words: cave amphipods; freshwater crustacean; morphology; taxonomy.

Introduction:

The transition from the surface to the underground presents many evident adaptations found in most animal groups (Trontelj *et al.*, 2012; Fišer *et al.*, 2013). Typical adaptations – called troglomorphies – include the loss of eyes and of pigmentation, and in some cases, the elongation of sensory appendages (Trontelj *et al.*, 2012). The amphipod genus *Hyalella* Smith, 1874 comprises both surface and underground species (Pereira, 1989; González *et al.*, 2006; Cardoso *et al.*, 2011), and differently from other genera of this order, not all cave species present evident troglomorphies (personal observation). Moreover, most *Hyalella* species are endemic to small areas, and even closely related water bodies can hold completely different *Hyalella* species (Rangel *et al.*, 2022).

Many new *Hyalella* species were described to Brazil in the last few years, mostly to the South region of the country (Talhaferro *et al.*, 2021; Limberger *et al.*, 2021, 2022; Rangel *et al.*, 2022). But there is still a great diversity to be recognized. *Hyalella caeca* Pereira, 1989 was the first cave species of the genus to be described to Brazil and the second overall (Pereira, 1989), but the original description lacks some important information. The species occurs at Tobias de Cima Cave, in the state of São Paulo, Southeast Brazil (Pereira, 1989).

Here we present two new species of the genus and redescribe *Hyalella caeca*, all found on the same river system – the Roncador River system in Parque Estadual Turístico do Alto Ribeira – PETAR, state of São Paulo, Brazil. There is a new cave species found inside of Santana Cave and a superficial (epigean) species, living just outside of the same cave; they morphologically distinct from one another and from *Hyalella* species previously described.

Material and methods:

Study area:

The Parque Estadual Turístico do Alto Ribeira – PETAR bears almost 500 documented caves (São Paulo, 2018). The vegetation all over the park is typical Atlantic Rainforest (Ferreira *et al.*, 2019) and the main river is the Ribeira de Iguapé, which gives the name to the region: Vale do Ribeira (or the Ribeira Valey). The climate is type “Cfa”, tropical without a dry season, with precipitations above 30 mm in July (the drier month) (Köeppen, 1948). The study area is inserted at the Upper Ribeira Valey’s karst area, in the state of São Paulo, Southeast Brazil. It is formed by the Upper Pre-Cambrian metasedimentary rocks and is part of the Açungui group (Karmann, 1994). The study area is crossed by typically well-oxygenated headwater streams, with rocky beds and rapids intercalated with some soft-bottomed pools, both outside and inside the caves (subterranean drainages) (Bichuette & Trajano, 2018). Here we describe species found on “Gruta Tobias” (a.k.a. “Gruta Tobias de Baixo”) and “Santana” (Fig. 1) caves. The Betari river belongs to the Ribeira de Iguapé water bay and is formed by the “Roncador” river affluent (Hassui *et al.*, 1992). The “Roncador” river is the one that forms Santana Cave (Lobo *et al.*, 2014) and connects it to the “Gruta Tobias” Cave. In all hypogean collected sites, *Hyalella* specimens showed preference of lentic and soft-bottomed stretches, and in the epigean collection site, the preference was for rocky and fast-flowing water.

The material from Gruta Tobias Cave (a.k.a. “Gruta Tobias de Baixo”) was collected in 1975 by Guy Christian, and not much description of collection site is available. It was deposited at the crustacean collection of Museu Nacional do Rio de Janeiro (MNRJ). The cave is part of the Pérolas-Santana system and has a high biological importance (CONSEMA, 2018).

The Santana Cave (official name: Caverna de Santana) is an important touristic cave of PETAR, with more than 8.5 kilometers in extension, but with less than 500m available for guided touristic visitation (São Paulo, 2018). Considered one of the biggest caves of Brazil, it is also amongst the most visited ones, with special need of attention to the impacts of tourism in its conservancy (Ferreira *et al.*, 2019).

Collection, identification, and species description

Collections at Santana Cave happened twice: first on November 2017 and later on February 2022, under authorization SISBIO/ICMBio 28992 and Instituto Florestal-SP 002914/2021-12. There were two collection sites. The first one was inside the cave (hypogean), on a lentic puddle (Fig. 2A). *Hyalella* individuals were swimming freely on the water and were collected using pipets directly from the pools were they occur, and placed on identified plastic tubes with 70% ethyl alcohol. The second collection site was on the outside of the cave (epigean), on a lotic river branch that exits the cave (Fig. 2B). The water current was heavy and *Hyalella* was collected using hand nets positioned against the water current, along some long plant roots, and placed on identified plastic tubes with 70% ethyl alcohol.

The material from Gruta Tobias cave was deposited at the Museu Nacional do Rio de Janeiro (MNRJcarcino) under collection number 7618. This material was preserved in alcohol 70% and we were able to reassign a new holotype (one entire male), one allotype (one entire female) and a new paratype (one male in slides). There is still some not well-preserved individuals together with the type material, but they are broken and/or unusable for morphological analysis.

For redescription of *Hyalella caeca* we used one paratype male from the original type material, which was deposited at the Crustacea Collection of Museu Nacional do Rio de Janeiro (MNRJcarcino).

We prepared semi-permanent slides following this protocol: 1) selected *Hyalella* males and females were stained using the “Red Congo” dye for 24h; 2) the animals were measured (head: from antenna insertion to end of head, before first thoracic segment; body: from antenna insertion to the end of the body, before telson insertion) under a stereomicroscope with an attached microscale; 3) slide preparation using fine insulin needles for appendage separation and glycerin as slide medium; 4) the slides were sealed with invisible painting varnish, for longer duration. These slides were latter used for appendage photographs using the Zen 2011 Zeiss Software, which served as a base for the drawings, produced with CorelDRAW X7, presented here.

Morphological description followed Zimmer *et al.* (2009) for cuticular structures and Rogers *et al.* (2020) for appendages. Type material of both new species were deposited at MNRJcarcino and at Coleção de Crustáceos da Universidade Federal de Lavras (CCUFLA).

Taxonomy

Order Amphipoda Latreille, 1816
Suborder Senticaudata Lowry & Myers, 2013
Family Hyalellidae Bulycheva, 1957
Genus *Hyalella* Smith, 1874
Hyalella sp2 n. sp. Penoni & Bueno (Figs. 3–6)

Type material: Holotype male (Fig. 3A), body length = 6.44 mm, head length = 0.53 mm, preserved in permanent slides (Canada Balsam as the mean), Santana Cave, Parque Estadual Turístico do Alto Ribeira, Iporanga municipality, São Paulo state, Brazil (24°31'51"S; 48°42'6"W), MNRJcarcino XXXXX, Nov. 2017, Bichuette, M.E. coll; allotype female (Fig. 3B), body length = 5.06 mm, head length = 0.5 mm, MNRJcarcino XXXX, Feb. 2022, Bichuette, M.E., Penoni, L.R., Zepon, T. coll.; paratype, one male in slides CCUFLA 457 and one female in slides CCUFLA 458 (same collection data as allotype).

Diagnosis: Body surface smooth. Epimeron 2 and 3 postero-distal margin acute. Eyes absent. Antenna 1 0.8× shorter than Antenna 2, longer than the ramus of Antenna 2; Antenna 2 more than half the body length. Maxilla 1 palp longer than wide, shorter than half the distance between the base of the palp and the base of setae on outer plate; outer plate with 8 serrate setae. Maxilliped heavily setose on the inner margin of palp and of outer ramus. Gnathopod 1 of male and female identical, propodus 1.3× longer than wide, hammer shaped, inner face with a row of seven papposerrate setae, with comb scales on the merus. Gnathopod 2 propodus ovate, palm subequal to posterior margin of propodus, slope oblique. Uropod 1 of the male without a curved seta on inner ramus. Uropod 3 longer than the telson, peduncle wider and shorter than the ramus. Telson almost square, no observed setae apically, two small plumose setae on each side, symmetrically. Coxal gills on segments 2–6. Sternal gills on segments 2–7.

Male: Mean body length 5.87 ± 0.81 mm ($N = 2$) (5.29 – 6.44 mm); mean head length 0.48 ± 0.07 mm ($N = 2$) (0.43 – 0.53 mm). Body surface smooth. Epimeral plates 2 and 3 accuminated (Fig. XJ). Coxae 1–3 similar, subequal in size, with some scattered microtrichs; coxa 4 as wide as long, excavated posteriorly. Eyes absent.

Antenna 1 (Fig. 4A) almost half the body length (0.4x), 0.8× shorter than Antenna 2, 2.3× longer than the peduncle of Antenna 2 and 1.2× longer than the flagellum of Antenna 2.

Peduncle articles 1–3 relative length 1:0.82:0.76; flagellum with 13 articles; aesthetascs occurring distally on flagellum after article 6.

Antenna 2 (Fig. 4B) 1.1× longer than half the body length; peduncle slender, double the length of the head; flagellum with 17 articles, 1.9× longer than the peduncle.

Mandible without palp; incisor toothed; left (Fig. 4C) *lacinia mobilis* with 4 teeth, setae row with 4 pappose setae and a few setules, no observed accessory seta on the molar process; right mandible (Fig. 4D) with 4 pappose setae and some setules; molar process cylindrical with an accessory seta.

Upper lip (Fig. 4E) margin rounded; distal border covered by setules. Lower lip (Fig. 4F) outer lobes rounded, distally notched, covered by setules apically.

Maxilla 1 (Fig. 4G) inner plate slender, with 2 apical pappose setae and lateral setules. Outer plate with 8 serrate setae, and a few setules on the inner margin, apically. Palp short, uniarticulate, longer than wide, reaching less than half the distance between base of the palp and base of the setae on the outer plate, with no apparent seta distally, only some short setules on the outer margin.

Maxilla 2 (Fig. 4H) inner plate shorter than outer plate, inner plate with 2 strong pappose setae distally and some simple seta disto-apically, and setules laterally; outer plate with longer simple setae and some papposerrate setae, disto-apically, and setules laterally.

Maxilliped (Fig. 4I) without comb scales; inner plate with 3 cuspidate distal setae apically, a few pappose setae disto-apically, 3 simple seta on the ventral margin, setules on the distal-outer margin and 2 simple setae on the outer margin; outer plate with several long simple setae on the inner margin and 2 smaller simple setae on the outer margin; palp longer than inner and outer plates, with 4 articles; article 1 inner margin with up to 4 long simple setae and outer margin with 1 simple setae; article 2 inner margin with several long simple setae and outer margin with 2 simple setae; article 3 inner margin with simple and papposerrate setae, and outer and distal margins with long simple setae; article 4 (dactylus) unguiform with distal nail present, with up to 6 simple setae, shorter than nail.

Gnathopod 1 (Fig. 5A) subchelate, identical on the male and the female; coxal plate 1.7× wider than long, with simple setae on the margin and a microtrich on the internal margin; basis with posterior and disto-posterior simple setae, with or without accessory seta; ischium with 1 apical simple seta; merus with serrate, papposerrate and simple seta on distal margin, and a slender row of comb scales distally; carpus longer than wide, with pappose setae on the lateral distal lobe, up to 5 pappose setae on inner margin and some simple or serrate setae distally; propodus 1.3× longer than wide, hammer shaped, 2 long simple setae with accessory seta on disto-posterior margin, some long and short simple setae on disto-anterior margin; palm slope transverse, with up to 10 long simple setae and 5 short simple setae; up to 6 pappose setae on inner face; dactylus claw-like, with a plumose seta distally.

Gnathopod 2 (Fig. 5B) subchelate, without comb scales or polygonal pattern; coxal plate 1.6× wider than long, with simple setae on the margin and some scattered microtrichs on the inner margin; basis with 5 long simple setae with accessory seta on posterior margin, 1 short simple setae on anterior margin, and up to 2 simple setae disto-posteriorly; ischium with 2 simple setae disto-posteriorly; merus with up to 6 serrate setae on distal margin; carpus lobe slim, produced between merus and propodus, posterior margin with pappose setae, inner margin with 2 small pappose setae and disto-anterior margin with 2 long serrate setae; propodus ovate, palm and posterior margin subequal in length, slope oblique, posterior margin with 2 simple setae, palm with a slight posterior excavation, some short cuspidate setae with accessory setae and some simple setae, anterior margin with a few simple setae; dactylus claw-like, congruent with palm and with one plumose seta distally.

Pereopods 3–7 (Fig. 5C–G) simple. Coxae with simple setae distally and microtrichs on inner face; coxa 3 similar to 1 and 2; coxa 4 as long as wide, excavated posteriorly; coxa 5 posterior lobe longer than anterior lobe; coxa 6 anterior lobe reduced; coxa 7 reduced. Pereopods 3 and 4 subequal in size, with 2 to 3 groups of 1 or 2 setae on the posterior margins of their basis, merus, carpus and propodus, a plumose seta on the dactylus. Pereopod 5 1.1× longer than P3 and P4, with groups of 1 to 2 cuspidate setae with accessory seta on the anterior margins of the basis, merus, carpus and propodus. Pereopod 6 1.3× longer than P5 and 0.9× the length of P7. Pereopods 6 and 7 with groups of 1 to 3 simple and/or cuspidate setae with accessory seta on the anterior margins of their basis, merus, carpus and propodus, a plumose seta on the dactylus.

Pleopods (Fig. 6A) peduncle 0.7× the length of the rami, no coupling spines observed; both rami with several long plumose setae.

Uropod 1 (Fig. 6B) peduncle 1.1× longer than inner ramus and 1.2× longer than the outer ramus, with 5 cuspidate setae with accessory seta and 2 short simple setae ; inner ramus subequal in size to outer ramus, slightly longer, with 3 cuspidate setae with accessory seta on dorsal margin and 5 cuspidate setae (2 longer, 1 shorter) apically, without curved seta; outer ramus with 3 cuspidate setae with accessory seta on dorsal margin and 4 cuspidate setae (1 longer, 3 shorter) apically.

Uropod 2 (Fig. 6C) 0.6× the total length of Uropod 1, 1.2× longer than the peduncle of Uropod 1, peduncle 0.7× the length of inner ramus, subequal to outer ramus, with 4 cuspidate setae with accessory seta and 1 short simple seta; inner ramus 1.3× longer than outer ramus, with 2 cuspidate setae with accessory seta on dorsal margin and 5 cuspidate setae (2 longer, 1 shorter) apically; outer ramus with 3 cuspidate setae with accessory seta on dorsal margin and 4 cuspidate setae (3 longer, 1 shorter) apically.

Uropod 3 (Fig. 6D) half the length of the peduncle of uropod 1 and subequal to the peduncle of uropod 2, 1.2× longer than the telson; peduncle with 2 cuspidate setae on outer distal margin; inner ramus absent; outer ramus with 3 cuspidate setae apically.

Telson (Fig. 6E) entire, as long as wide, apically rounded and with no apparent setae; 2 small plumose setae on each side, symmetrically.

Epimeron (Fig. 4J) 2 and 3 postero-distal margin acute; first and third epimeron shorter than the second one.

Coxal gills sac-like on pereonites 2-6. Sternal gills tubular on pereonites 2-7.

Female: (Fig. 6F–G) Mean body length 5.54 ± 0.67 mm (*N* = 2) (5.06 – 6.01 mm); mean head length 0.49 ± 0.01 mm (*N* = 2) (0.49 – 0.5 mm). Gnathopod 1 identical to the male's. Gnathopod 2 (Fig. 6F) similar in size and shape to Gnathopod 1; merus with 6 long papposerrate setae; carpus with 2 pappose seta on the inner face; propodus elongated, with several long simple setae on the inner face and short simple setae on the palm; palm transverse; dactylus claw-like with a distal plumose seta. Telson (Fig. 6G) similar to the male's, but with an observed cuspidate seta apically, and 3 small plumose setae laterally on each side, symmetrically.

Habitat: Freshwater, hypogean, troglobite, stygobite. A few specimens of *Hyalella sp2* n. sp. were found swimming freely or burying themselves on the sandy bottom of the lentic pools of "Caverna Santana".

Conservation: H. sp2 n. sp. is classified as endangered (EN) based on IUCN subcategory B1 (< 5.000km^2 in area of occurrence extension) and D1 (< 250 mature individuals) (ICMBIO, 2013).

Remarks: Hyalella sp2 n. sp. is hypogean with two important adaptations to the life underground: the absence of eyes and loss of pigmentation. These features can be used to differentiate it from mostly of the known *Hyalella* species. It is similar, however, to other cave species that are also blind and unpigmented, namely: *H. caeca*, *H. epikarstica* Rodrigues, Bueno & Ferreira, 2014 and *H. formosa* Cardoso & Araujo, 2014 (Cardoso *et al.*, 2014). Table 1 presents highlights on the morphological characteristics of subterranean and troglomorphic *Hyalella* species. There is also *H. veredae* Cardoso & Bueno, 2014 (Araújo *et al.*, 2014) and *H. speleae* Bueno & Cardoso, 2011 (Cardoso *et al.*, 2011), but in these cases, the eyes can be either present, reduced or absent on these species populations. *Hyalella sp2* n. sp. can be set apart from *H. formosa* and *H. epikarstica* because the new species has Antenna 1 longer than Antenna 2, while for the other 2 it is the opposite. There are several differences between *H. sp2* n. sp. and *H. caeca*, which will be detailed latter on this paper.

Hyalella sp3 n. sp. Penoni & Bueno (Figs. 7–10)

Type material: Holotype male (Fig. 7A), body length = 8.81 mm, head length = 0.78 mm, Santana Cave entrance, Parque Estadual Turístico do Alto Ribeira, Iporanga municipality, São Paulo state, Brazil (24°31'51"S; 48°42'6"W), MNRJcarcino XXXXX, Feb. 2022, Bichuette, M.E., Penoni, L.R., Zepon, T. coll.; allotype female (Fig. 7B), body length = 8.71 mm, head length = 0.73 mm, MNRJcarcino XXXX; paratype 20+ hole individuals CCUFLA 461, one male in slides CCUFLA 459 and one female in slides CCUFLA 460 (same collection data as holotype).

Diagnosis: Body surface smooth. Epimeron 2 and 3 postero-distal margin acute. Eyes present, normal. Antenna 1 half the size of Antenna 2; Antenna 2 more than half the body length. Maxilla 1 outer plate with 9 serrate setae. Gnathopod 1 of male and female identical, propodus 1.4× longer than wide, with polygonal pattern on the disto-posterior margin of the propodus. Gnathopod 2 propodus ovate with a slender posterior excavation on the palm. Uropod 1 of the male without a curved seta on inner ramus. Uropod 3 ramus with an excavation midway to the apex, with up to 3 setae. Telson longer than wide. Coxal gills on segments 2–6. Sternal gills on segments 2–7.

Male: Mean body length 9.32 ± 0.46 mm (*N* = 3) (8.81 – 9.69 mm); mean head length 0.84 ± 0.06 mm (*N* = 3) (0.78 – 0.89 mm). Body surface smooth. Epimeral plates 2 and 3 accuminated (Fig. XJ). Coxae 1–3 similar, subequal in size, with some scattered microtrichs on the anterior margins on coaxes 2 and 3; coxa 4 as wide as long, excavated posteriorly, with microthrichs. Eyes present, rounded and large.

Antenna 1 (Fig. 8A) 0.3× the body length, 0.56× the length of Antenna 2, 1.5× longer than the peduncle of Antenna 2 and 0.9× the length of the flagellum of Antenna 2. Flagellum with 19 to 21 articles; aesthetascs occurring distally on flagellum after article 5.

Antenna 2 (Fig. 8B) longer than half the body length (0.6× the body length); peduncle slender, 2.3× the length of the head; flagellum with 27 articles, 1.8× longer than the peduncle.

Mandible without palp; incisor toothed; left (Fig. 8C) *lacinia mobilis* with 6 teeth, setae row with 3 pappose setae and a few setules, accessory seta on the molar process; right mandible (Fig. 8D) with 4 pappose setae and some setules; molar process cylindrical with a small accessory seta.

Upper lip (Fig. 8E) margin rounded; distal border covered by setules and a few scattered setules laterally. Lower lip (Fig. 8F) outer lobes rounded, distally notched, covered by setules apically.

Maxilla 1 (Fig. 8G) inner plate slender, with 2 apical pappose setae and lateral setules. Outer plate with 9 serrate setae, a few setules on the inner margin, apically. Palp short, uniarticulate, longer than wide, reaching half the distance between base of the palp and base of the setae on the outer plate, with one long serrate seta apically.

Maxilla 2 (Fig. 8H) inner plate shorter than outer plate, inner plate with 1 strong pappose setae distally and some simple and papposerrate seta disto-apically, scattered setules laterally; outer plate with longer simple setae and one papposerrate setae, disto-apically, scattered setules laterally.

Maxilliped (Fig. 8I) without comb scales; inner plate with 3 large cuspidate distal setae apically, up to 15 pappose setae disto-apically, setules on the distal-outer margin, 4 simple setae on the outer margin; outer plate with several long simple setae with accessory seta on the inner margin, 3 simple seta on the inner face, and up to 5 simple setae on the outer margin; palp subequal to outer plate and shorter than inner plate, with 4 articles; article 1 inner margin with 2 simple setae with accessory seta and outer margin with 4 simple setae and 1 plumose seta; article 2 inner and disto-inner margins with several simple setae with accessory seta and disto-outer margin with 3 long and 2 simple setae and 1 long plumose seta; article 3 inner margin, outer and distal margins with long simple setae; article 4 (dactylus) unguiform with distal nail present, with up to 4 simple setae.

Gnathopod 1 (Fig. 9A) subchelate, identical on the male and the female; coxal plate 1.8× wider than long, with simple setae on the anterior margin, 5 serrate setae on the posterior inner face, close to the attachment of the basis; basis with posterior and disto-posterior serrate setae; ischium with 2 apical simple setae; merus with 6 papposerrate seta on distal margin; carpus longer than wide, with pappose setae on the lateral distal lobe, up to 4 papposerrate setae on inner face and up to 7 serrate setae distally; propodus 1.4× longer than wide, hammer shaped, up to 9 pairs of simple setae on the posterior margin, with polygonal pattern on the disto-posterior margin and some long and short simple setae on disto-anterior margin; palm slope transverse, with several long and short simple setae; 8 long pappose setae and some smaller ones on inner face; dactylus claw-like, small simple setae on the proximal margin, with a plumose seta distally.

Gnathopod 2 (Fig. 9B) subchelate; coxal plate 1.7× wider than long, with simple setae on the margin, 2 simple setae on the posterior inner face, close to the attachment of the basis, and some scattered microtrichs on the inner face; basis with 5 to 8 serrate setae on posterior margin, 1 short simple setae on anterior margin, and 3 serrate setae disto-posteriorly; ischium with 4 serrate setae disto-posteriorly; merus with up to 4 serrate setae and 3 simple setae with accessory seta on distal margin; carpus lobe slim, produced between merus and propodus, posterior margin with several pappose setae, disto-posterior corner with polygonal pattern, inner margin with some scattered simple and plumose setae, and disto-anterior margin with 4 serrate setae; propodus ovate, slope oblique, posterior margin with 5 groups of 1 to 3 simple setae, palm with a slight posterior excavation, two rows of short cuspidate setae with accessory setae and simple setae, anterior margin with several simple setae, inner face with a row of short simple setae; dactylus claw-like, congruent with palm, with small simple setae on the proximal margin and a plumose seta distally.

Pereopods 3–7 (Fig. 9C–G) simple. Coxae 3–7 with simple setae distally and microtrichs on inner face; coxa 3 similar to 1 and 2; coxa 4 as long as wide, excavated posteriorly; coxa 5 posterior lobe as long as anterior lobe; coxa 6 anterior lobe reduced; coxa 7 reduced. Pereopods 3 and 4 subequal in size, with 3 to 4 groups of 1 or 2 serrate setae on the posterior margins of their basis and on their basis and ischium disto-posterior margins, merus with 4 to 6 groups of 1 to 4 simple setae on the posterior margin, disto-anterior and disto-posterior margin with 4 to 7 serrate setae, carpus and propodus with 6 to 8 groups of up to 5 cuspidate setae with accessory seta and simple setae on the posterior margin, a plumose seta on the dactylus. Pereopod 5 is the shorter, 0.9× the length of P3 and P4 and 0.7× the length of P6 and P7, with groups of 1 to 3 cuspidate setae with accessory seta on the anterior margin of the basis and posterior margin of the merus, and groups of cuspidate setae with accessory seta and simple setae on the disto-posterior and disto-anterior margins of basis, ischium, merus, carpus and propodus, and the anterior margins of merus, carpus and propodus, a plumose setae on the dactylus. Pereopod 6 subequal in length to P7, longer than P3–P5. Pereopods 6 and 7 with 3 to 5 serrate setae on the proximo-anterior margin of basis, and groups of 1 to 5 cuspidate setae with accessory seta on the anterior margins of their basis, merus, carpus and propodus and posterior margin of merus, a plumose seta on the dactylus of pereopod 6.

Pleopods (Fig. 10A) peduncle half the length of the rami, 2 coupling spines; both rami with several long plumose setae.

Uropod 1 (Fig. 10B) peduncle 1.2× longer than inner ramus and 1.4× longer than the outer ramus, with 5 cuspidate setae with accessory seta; inner ramus 1.2× longer than the outer ramus, with 2 cuspidate setae with accessory seta on dorsal margin and 6 cuspidate setae (2 longer, 2, medium and 2 shorter) apically, without curved seta; outer ramus with 3 cuspidate setae on dorsal margin and 4 cuspidate setae (2 longer, 2 shorter) apically.

Uropod 2 (Fig. 10C) 0.6× the total length of Uropod 1, 1.2× longer than the peduncle of Uropod 1, peduncle subequal in length to the inner ramus, peduncle and inner ramus 1.1× longer than the ramus, peduncle with 4 cuspidate setae with accessory seta disto-laterally; inner ramus with 3 cuspidate setae with accessory seta, growing in size, on dorsal margin and 6 cuspidate setae (2 longer, 2 medium with accessory seta, and 2 shorter) apically; outer ramus with 3 cuspidate setae with accessory seta on dorsal margin and 4 cuspidate setae (2 longer, 2 shorter) apically.

Uropod 3 (Fig. 10D) half the length of the peduncle of uropod 1 and 0.8× the length of peduncle of uropod 2, subequal to the telson; peduncle with up to 5 cuspidate setae with accessory seta on outer distal margin; inner ramus absent; outer ramus with 3 cuspidate setae with or without accessory seta halfway to the apex, and up 6 cuspidate setae, with or without accessory seta, apically.

Telson (Fig. 10E) entire, longer than wide, apically rounded and with two short cuspidate setae; 3 small plumose setae on each side, symmetrically.

Epimeron (Fig. 8J) 1 postero-distal margin rounded, 2 and 3 postero-distal margin acute; epimeron 2 longer than 1 and 3.

Coxal gills sac-like on pereonites 2-6. Sternal gills tubular on pereonites 2-7.

Female: (Fig. 10F–G) Mean body length 8.12 ± 0.88 mm (*N* = 3) (7.11 – 8.71 mm); mean head length 0.74 ± 0.11 mm (*N* = 3) (0.63 – 0.85 mm). Gnathopod 1 identical to the male's. Gnathopod 2 (Fig. 10F) similar in size and shape to Gnathopod 1; basis posterior and distal margins, ischium and merus posterior margin with simple and serrate setae; carpus with 2 pappose seta on the inner face; propodus with a row of simple and pappose setae on the inner margin; palm transverse, 0.6× the length of posterior margin, with a row of short simple setae and another of longer simple setae, polygonal pattern on the disto-posterior margin; dactylus claw-like with a distal plumose seta. Telson (Fig. 10G) identical to the male's.

Habitat: Freshwater, epigean. Specimens of *Hyalella sp3* n. sp. were found associated to plant roots just outside of "Caverna Santana", on the rapids of the stream.

Conservation: H. sp3 n. sp. is classified as vulnerable (VU) based on IUCN subcategory D2 (restricted area of occurrence) (ICMBIO, 2013). It is worth mentioning that the species is possibly endemic.

Remarks: Hyalella sp3 n. sp. can be easily distinguished from *H. sp2* n. sp. and *H. caeca* and all other subterranean *Hyalella* because it lacks the typical adaptations to life underground. Table 2 highlights the morphological characteristics of superficial (*i. e.* non-subterrenean) *Hyalella* species that occur in Brazil with some morphological similarities to *H. sp3* n. sp. There are two characteristics easily used to group *Hyalella* and, hence, differentiate species: the presence/absence of a curved seta on the inner ramus of male’s uropod 1; and the presence/absence/position of flanges (a dorsoposterior carina on the thoracic and abdominal plates). *Hyalella sp3* n. sp. has neither a curved seta, which differentiate it from 21 Brazilian species, nor flanges, which differentiate it from other 1 species from Brazil. The other remaining 9 species can be found on Table 2, in comparison to *Hyalella sp3* n. sp.

Hyalella caeca Pereira, 1989 (Figs. 11–14)

Type material: Holotype male (Fig. 11A), body length = 5.79 mm, head length = 0.50 mm, “Gruta Tobias” Cave (a.k.a. “Gruta Tobias de Baixo”), Parque Estadual Turístico do Alto Ribeira, Iporanga municipality, São Paulo state, Brazil (24°33'25"S; 48°43'25"W), MNRJcarcino 7618, Novembrer 06 1975, Guy Christian coll.; allotype female (Fig. 11B), body length = 4.92 mm, head length = 0.42 mm, MNRJcarcino 7618; paratype male in slides (same collection data as holotype).

Diagnosis: Body surface smooth. Epimeron 2 postero-distal margin acute. Eyes absent. Antenna 1 0.3× the body length; Antenna 2 almost half the body length. Maxilla 1 outer plate with 9 serrate setae. Male gnathopod 1 propodus 1.6× longer than wide, hammer shaped, inner face with a row of 6 papposerrate setae, with polygonal pattern on the dactylus. Gnathopod 2 propodus almost triangular, palm with a slender excavation posteriorly. Uropod 1 of the male without a curved seta on inner ramus. Uropod 2 1.1× longer than the peduncle of Uropod 1. Uropod 3 subequal in length to the peduncle of Uropod 2 and the telson. Telson almost square, 2 setae apically, 3 small plumose setae on each side, symmetrically. Coxal gills on segments 2–6. Sternal gills on segments 2–7.

Male: Mean body length 5.79 ± 0.86 mm (*N* = 2) (5.79 – 7.00 mm); mean head length 0.5 ± 0.06 mm (*N* = 2) (0.5 – 0.59 mm). Body surface smooth. Epimeral plate 2 accuminated (Fig. XI). Coxae 1–3 similar, subequal in size; coxa 7 reduced. Eyes absent.

Antenna 1 (Fig. 12A) short, only 0.3× the body length and 0.7× the length of Antenna 2, 1.8× longer than the peduncle of Antenna 2 and slightly (1.1×) longer than the flagellum of Antenna 2. Peduncle articles 1–3 relative length 0.9:1:0.7; flagellum with 10–11 articles; aesthetascs occurring distally on flagellum after article 4.

Antenna 2 (Fig. 12B) almost (0.88×) half the body length; peduncle slender, double the length of the head; flagellum with 13–14 articles, 1.4× longer than the peduncle.

Mandible without palp; incisor toothed; left (Fig. 12C) *lacinia mobilis* with 5 teeth, setae row with 3 pappose setae and a few setules, no observed accessory seta on the molar process; right mandible (Fig. 12D) with 2 pappose setae and some setules; molar process cylindrical with no observed accessory seta.

Upper lip (Fig. 12E) margin rounded; distal border covered by setules. Lower lip (Fig. 12F) outer lobes rounded, distally notched, covered by setules apically.

Maxilla 1 (Fig. 12G) inner plate slender, with 2 apical pappose setae and a few lateral setules. Outer plate with 9 serrate setae, and a few setules on the inner margin, apically. Palp short, uniarticulate, longer than wide, reaching less than half the distance between base of the palp and base of the setae on the outer plate, with 1 short simple seta distally.

Maxilla 2 (Fig. 12H) inner plate shorter than outer plate, inner plate with 1strong pappose setae distally and some simple and serrate seta disto-apically; outer plate with longer simple setae and some papposerrate setae, disto-apically.

Maxilliped (Fig. 14A) without comb scales; inner plate (Fig. 14A1) with 3 cuspidate distal setae apically, a few pappose setae disto-apically and on the ventral margin, 2 simple setae on the outer margin; outer plate (Fig. 14A2) with several simple setae on the inner margin, 2 setae on the inner face and 2 smaller simple setae on the outer margin; palp (Fig. 14A3–6) longer than inner and outer plates, with 4 articles; article 1 (Fig. 14A3) inner margin with up to 4 long simple setae and outer margin with 1 simple setae; article 2 (Fig. 14A4) inner margin with several long simple setae and outer margin with 3 simple setae; article 3 (Fig. 14A5) inner, distal and outer margins with several simple setae; article 4 (Fig. 14A6 – dactylus) unguiform with distal papposerrate nail present, with up to 5 simple setae, shorter than nail.

Gnathopod 1 (Fig. 13A) subchelate; coxal plate 1.5× wider than long, with simple setae on the margin; basis posterior and disto-posterior margins with bifid setae; ischium with 1 disto-posterior simple seta; merus with 3 simple setae distally, and up to 4 bifid setae disto-posteriorly; carpus longer than wide, with pappose setae on the lateral distal lobe, up to 6 pappose setae on inner margin and some serrate setae distally; propodus 1.6× longer than wide, hammer shaped, few simple or bifid setae on disto-posterior margin, 2 rows of small comb scales on the disto-posterior corner; up to 16 long and short simple setae on disto-anterior margin; inner face with a row of 6 papposerrate setae and some scattered shorter simple setae; palm slope transverse, with up to 15 simple setae; dactylus claw-like, with a plumose seta distally, polygonal pattern on the distal margin and short simple setae on the anterior margin.

Gnathopod 2 (Fig. 13B) subchelate, without comb scales or polygonal pattern; coxal plate 1.5× wider than long, with simple setae on the margin; basis with up to 5 long bifid setae on posterior margin, 1 short simple setae on anterior margin, and up to 2 bifid setae disto-posteriorly; ischium with 1 long simple setae disto-posteriorly; merus with up to 5 bifid setae on distal margin; carpus lobe slim, produced between merus and propodus, posterior margin with pappose setae, inner margin with 2 small pappose setae and disto-anterior margin with 2 long bifid setae; propodus almost triangular, palm slightly longer than posterior margin, slope oblique, with a slender posterior excavation, some short cuspidate setae with accessory setae, bifid setae and simple setae, anterior margin with a 4 short simple setae; dactylus claw-like, with one plumose seta distally.

Pereopods 3–7 (Fig. 13C–G) simple. Coxae with simple setae; coxa 3 similar to 1 and 2; coxa 4 1.2× longer than wide, excavated posteriorly; coxa 5 posterior lobe longer and narrower than anterior lobe; coxa 6 anterior lobe reduced; coxa 7 reduced. Pereopods 3, 4 and 5 subequal in size, pereopods 3 and 4 with 2 to 3 groups of 1 to 2 bifid or simple setae on the posterior margins of their basis, merus and carpus, 2 bifid setae on the anterior margin of their merus, propodus with 4 to 5 groups of 1 to 3 simple or cuspidate setae on the posterior margin, and a plumose seta on the dactylus. Pereopod 6 and 7 subequal in size, ~1.2× longer than P3–P5; Pereopods 5–7 with 2 to 6 groups of 1 to 3 simple, bifid or cuspidate setae with accessory seta on the anterior margins of the basis, merus, carpus and propodus, 2 bifid or cuspidate setae with accessory seta on the posterior margin of the merus, groups of 3 to 6 bifid or cuspidate setae with accessory seta on the disto-anterior and disto-posterior margins of merus and carpus.

Pleopods (Fig. 14B) peduncle 0.7× the length of the rami, a pair of coupling spines; both rami with several long plumose setae.

Uropod 1 (Fig. 12J) peduncle 1.3× longer than inner ramus and 1.4× longer than the outer ramus, with 5 cuspidate setae with accessory seta; inner ramus slightly longer than outer ramus, with 3 cuspidate setae with accessory seta on dorsal margin and 6 cuspidate setae (3 shorter) apically, without curved seta; outer ramus with 3 cuspidate setae with accessory seta on dorsal margin and 4 cuspidate setae (2 longer, 2 shorter) apically.

Uropod 2 (Fig. 12K) 0.6× the total length of Uropod 1, 1.1× longer than the peduncle of Uropod 1, peduncle 0.8× the length of inner ramus and 0.9× the length of outer ramus, with 4 cuspidate setae with accessory seta; inner ramus 1.1× longer than outer ramus, with 3 cuspidate setae with accessory seta on dorsal margin and 5 cuspidate setae apically; outer ramus with 3 cuspidate setae with accessory seta on dorsal margin and 4 cuspidate setae (3 longer, 1 shorter) apically.

Uropod 3 (Fig. 12L) almost half the length of the peduncle of uropod 1 and subequal to the peduncle of uropod 2 and to the telson; peduncle with 4 cuspidate setae on outer distal margin; inner ramus absent; outer ramus with 4 cuspidate setae (2 longer, 2 shorter) apically.

Telson (Fig. 12M) entire, as long as wide, apically rounded and with 2 apical cuspidate setae and 3 small plumose setae on each side, symmetrically.

Epimeron (Fig. 12I) 1, 2 and 3 postero-distal margins rounded, acute and sub-acute, respectively.

Coxal gills sac-like on pereonites 2-6. Sternal gills tubular on pereonites 2-7.

Habitat: Freshwater, hypogean, troglobite, stygobite.

Conservation: H. caeca is classified as endangered (EN) based on IUCN subcategory B1 (< 5.000km^2 in area of occurrence extension) and D1 (< 250 mature individuals) (ICMBIO, 2013).

Remarks: Hyalella caeca specimens were collected in 1975 inside of “Gruta Tobias de Baixo” Cave and described in 1989 (Pereira, 1989). It was the first hypogean species of the genus known to Brazil and second worldwide. On its original description, the author briefly compared its morphology with *Hyalella anophthalma* Ruffo, 1957, pinpointing the absence of eyes and pigmentation, elongation of the antennas, reduced number of setae on a narrow ramus on the uropod 3 and the morphology of the telson (Pereira, 1989). Here we observe that the antennas are not longer than those of other species on average for the genus, and hence cannot be treated as a troglomorphy. When we compare *H. caeca* with *H. sp2* n. sp., the main differences are the relative size of antenna 1 with the body length, the relative size of articles 1–3 of the peduncle of antenna 1, the number of serrate setae on the outer plate of maxilla 1, the presence of comb scales and polygonal pattern on the male gnathopod 1 of *H. caeca* and the relative length of the pereopods. Table 1 indicates other differences between *H. caeca* and *H. sp2* n. sp. and other subterranean *Hyalella* species. It is worth mentioning that the material analyzed was indicated at the description of *H. spelaea*, but these are not the same species.

Final considerations

In this paper we describe a new subterranean and a new superficial species of the genus *Hyalella*. We hypothesize a parapatric evolution since both populations are found on the same river and on close proximity. *Hyalella sp2* n. sp. occurs inside of Santana Cave, on lentic patches of water, swimming freely and hiding on the sandy substrate. It presents typical troglomorphies, such as absence of eyes and of pigmentation. On the other hand, *H. sp3* n. sp. is found just outside of the same cave, on the rapids of the water body that exits the cave, associated to plant roots. Their morphologies are comparably different which makes it easy to separate them.

Here we also redescribe *Hyalella caeca*, which was firstly presented more than three decades ago (Pereira, 1989). The original description, although detailed for the time it was published, lacks some important information on appendages' morphologies. We increment these details and compare the two new species and other species known to Brazil with this one, the very first cave *Hyalella* species to be known to Brazil. We used the material designated as paratype from the 1989 description. On Pereira's paper, there is no deposit number indicated, and considering the 2018 fire that destroyed some of the collections and paperwork of Museu Nacional, we don't know the whereby of the holotype. Hence, we here designated a new holotype. It is important to note that, although Cardoso *et al.* (2011) indicated the examination of

MNRJcarcino 7618 material (the one used here) for the description of *Hyalella spelaea*, *H. caeca* and *H. spelaea* are **not** the same species.

Both *Hyalella caeca*, *H. sp2* n. sp. and *H. sp3* n. sp. are connected through the “Roncador” river system (Ayub, 1998), but these species are morphologically different from one another. Moreover, many caves from PETAR and from other parks nearby (*e. g.* Parque Estadual Intervales – PEI; and Parque Estadual da Caverna do Diabo) where sampled in the past few years (personal communication and collections), with the observation of *Hyalella* populations on many of these caves. Future analyses are due, but preliminary observations indicate that most of them are of new species. Furthermore, the growth on the number of new species of the genus, most of which are restricted to the type material`s collection sites, suggests that tropical *Hyalella* are generally endemic to short geographical ranges. Thus, we can expect that new populations found on collections sites never explored (for *Hyalella*) are most certainly of a new species.

References

Ayub, S. (1998). *Aplicação do corante traçador fluorescente rhodamina-wt no estudo hidrológico e hidrogeológico dos sistemas carsticos Pérolas-Santana, Grilo e Zezo, Município de Iporanga, Estado de São Paulo*. Doctoral dissertation, Universidade de São Paulo, São Paulo, Brazil, p. 117.

Bichuette, M. E.; Trajano, E. (2018). Diversity of *Potamolithus* (Littorinimorpha, Truncatelloidea) in a high- diversity spot for troglobites in southeastern Brazil: role of habitat fragmentation in the origin of subterranean fauna, and conservation status. *Subterranean Biology, 88*: 61–88.

Bulycheva, A. (1957) The sea fleas of the USSR and adjacent waters (Amphipoda: Talitroidea). Keys to the Fauna of the USSR. *Akademiia Nauk SSSR, Opredeliteli po Faune SSSR, 65*, 1–185 (in Russian).

Camargo, A. & Brandi, R. (2012). Exploração e documentação das cavidades naturais subterrâneas das regiões de Bulha d'Água, Vale dos Buenos, Fundão, Caboclos e entornos (Parque Estadual Turístico do Alto Ribeira-PETAR e Parque Estadual de Intervales–PEI), estado de São Paulo. *ESPELEO-TEMA*, 103–115.

Cardoso, G. M., Araújo, P. B., Bueno, A. A.P., & Ferreira, R. (2014). Two new subterranean species of *Hyalella* Smith, 1874 (Crustacea: Amphipoda: Hyalellidae) from Brazil. *Zootaxa, 3814(3)*, 353-368.

Cardoso, G. M., Bueno, A. A. D. P. & Ferreira, R. L. (2011). A new troglobiotic species of *Hyalella* (Crustacea, Amphipoda, Dogielinotidae) from Southeastern Brazil. *Nauplius*, 19, 17-26.

CONSEMA, Conselho Estadual do Meio Ambiente. (2018). Anexo 3 do Relatório da CTBio de 27/04/2018 (aprovado na 68ª reunião): Resumo Executivo - versão 2018. 191p.

Ferreira, A. R. R., Lobo, H. A. S. & de Jesus Perinotto, J. A. (2019). Inventory and quantification of geosites in the state tourist park of Alto Ribeira (PETAR, São Paulo state, Brazil). *Geoheritage*, 11(3), 783-792.

Fišer, C., Zagmajster, M. & Zakšek, V. (2013). Coevolution of life history traits and morphology in female subterranean amphipods. *Oikos*, 122(5), 770-778.

González, E. R., Bond-Buckup, G. & Araujo, P. B. (2006). Two new species of *Hyalella* from Southern Brazil (Amphipoda: Hyalellidae) with a taxonomic key. *Journal of Crustacean Biology*, 26(3), 355-365.

Hassui, Y., Coppedê-Junior, A., Caetano, A. C. & Sanchez, L. H. (1992). Aspectos geológico-estruturais da área de Furnas (Município de Iporanga, SP) e o problema do fluxo de efluentes. In Hasui, Y. e Mioto, J. A. (Coord), Geologia Estrutural Aplicada, São Paulo: Associação Brasileira de Geologia e Engenharia: Votorantim, p. 399–410.

ICMBIO (Instituto Chico Mendes de Conservação da Biodiversidade). (2013). *Aplicação de critérios e categorias da UICN na avaliação da fauna brasileira.* Version 2.0. ICMBIO, Brasilia, Brazil.

Karmann, I. (1994). *Evolução e dinâmica atual do sistema cárstico do Alto Vale do Rio Ribeira de Iguape, Sudeste do Estado de São Paulo*. São Paulo. Phd. Thesis – Instituto de Geociências, Universidade de São Paulo, São Paulo, Brasil.

Köeppen, W. (1948). *Climatología*. Fondo de cultura economica, Mexico. 478 pp.

Latreille, P. A. (1816) Amphipoda. In: *Nouveau Dictionaire d'histoire naturelle, appliquée aux Arts, à l'Agriculture, à l'Économie rurale et domestique, à la Médecine, etc. Par une société de Naturalistes et d'Agriculteurs.* 2nd edition. Volume 1. Deterville, Paris. Pp. 467–469.

Limberger, M. & Graichen, D. N. S. (2021). A new species of freshwater amphipod (Crustacea, Peracarida, Hyalellidae) from Southern Brazil. *Zootaxa*, 5026(2), 182-200.

Limberger, M., Santos, S. & Castiglioni, D. D. S. (2022). *Hyalella luciae* (Crustacea, Amphipoda, Hyalellidae) – a new species of freshwater amphipod from Southern Brazil. *Zootaxa*, 5174(5), 568-582.

Lobo, H. A. S. (2008). Capacidade de Carga Real (CCR) da Caverna de Santana, Parque Estadual Turístico do Alto Ribeira (PETAR)-SP, e indicações para o seu manejo turístico. *Geociências,* 369-385.

Lowry, J. K., & Myers, A. A. (2013). A phylogeny and classification of the Senticaudata subord. nov. Crustacea: Amphipoda. *Zootaxa, 3610(1)*, 1-80.

Penoni, L. R., Bueno, A. A. P. & Bichuette, M. E. *in press*. Two new species of *Hyalella* Smith, 1874 (Amphipoda: Hyalellidae) from Southeast Brazil, with a pictorial key to the hypogean species of the genus.

Pereira, V. F. G. C. (1989). Uma nova espécie de anfípode cavernícola do Brasil: *Hyalella caeca* n. sp. (Amphipoda, Hyalellidae). *Revista Brasileira de Zoologia*, 6, 49-55.

Rangel, C., Silva, A. L. L., Siegloch, A. E., Limberger, M. & Castiglioni, D. D. S. (2022). First island species of *Hyalella* (Amphipoda, Hyalellidae) from Florianpolis, state of Santa Catarina, Southern Brazil. *Zootaxa*, 5116(1), 40-60.

Rodrigues, S.G., Bueno, A.A.P. & Ferreira, R.L. (2012) The first hypothelminorheic Crustacea (Amphipoda, Dogielinotidae, *Hyalella*) from South America. *ZooKeys*, *236*, 65–80. https://doi.org/10.3897/zookeys.236.3930.

Ruffo, S. (1957). Una nuova specie troglobia di *Hyalella* del Venezuela (Amphipoda, Talitridae). (Viaggio in Venezuela di Nino San filippo IV). *Ann. Museo Civico Storia Nat. Genova*, *69*, 363-369.

São Paulo, Plano de Manejo – Parque Estadual Turístico do Alto Ribeira – PETAR, 2018. 209p.

Smith, S.I. 1874. The Crustacea of the freshwaters of the United States. A synopsis of the higher freshwater Crustacea of the Northern United States, Appendix F. Natural History. *United States Commission of Fish and Fisheries, 2*, 637–661.

Talhaferro, J. T., Bueno, A. A. P., Pires, M. M., Stenert, C., Maltchik, L. & Kotzian, C. B. (2021). Three new species of *Hyalella* (Crustacea: Amphipoda: Hyalellidae) from the Southern Brazilian Coastal Plain. *Zootaxa*, 4970(2), 257292.

Trontelj, P., Blejec, A. & Fišer, C. (2012). Ecomorphological convergence of cave communities. *Evolution: International Journal of Organic Evolution*, 66(12), 3852-3865.

Figures:

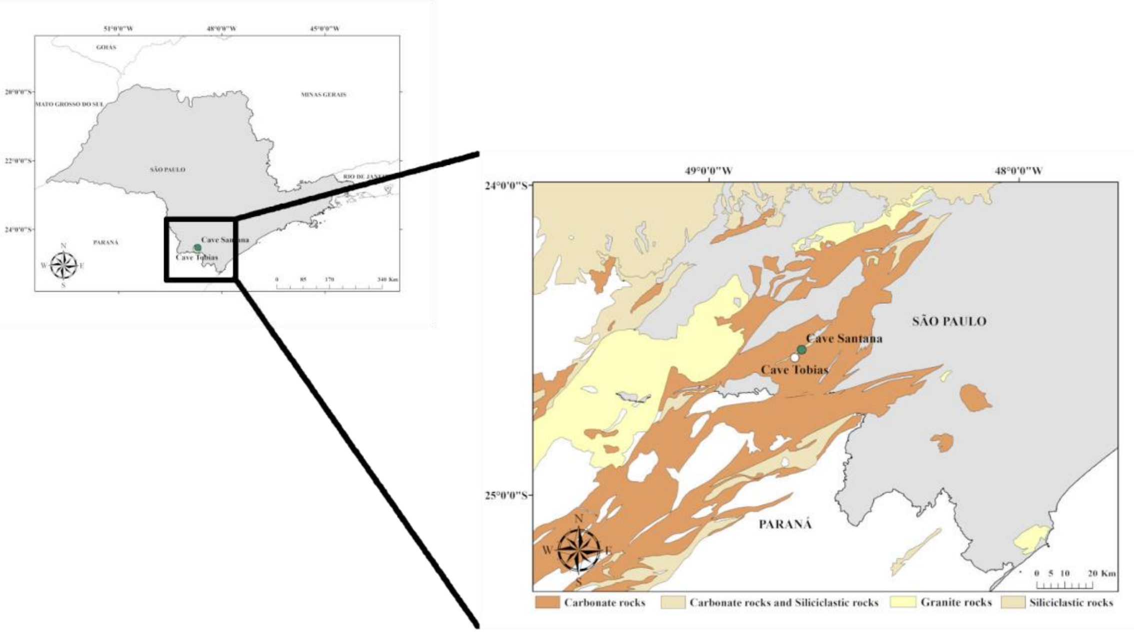

Figure 1: Map of collection sites of *Hyalella sp2* n. sp. and *H. sp3* – Santana Cave, Parque Estadual Turístico do Alto Ribeira; and of *H. caeca* – “Gruta Tobias de Baixo” Cave, municipality of Iporanga, state of São Paulo.

Figure 2: Collection sites of *Hyalella sp2* n. sp. and *H. sp3* n. sp. – Santana Cave, municipality of Iporanga, state of São Paulo, Brazil. Subterranean pool – type locality of *H. sp2* n. sp. (**A** and **B**); epigean rapids – type locality of *H. sp3* n. sp. (**C** and **D**).

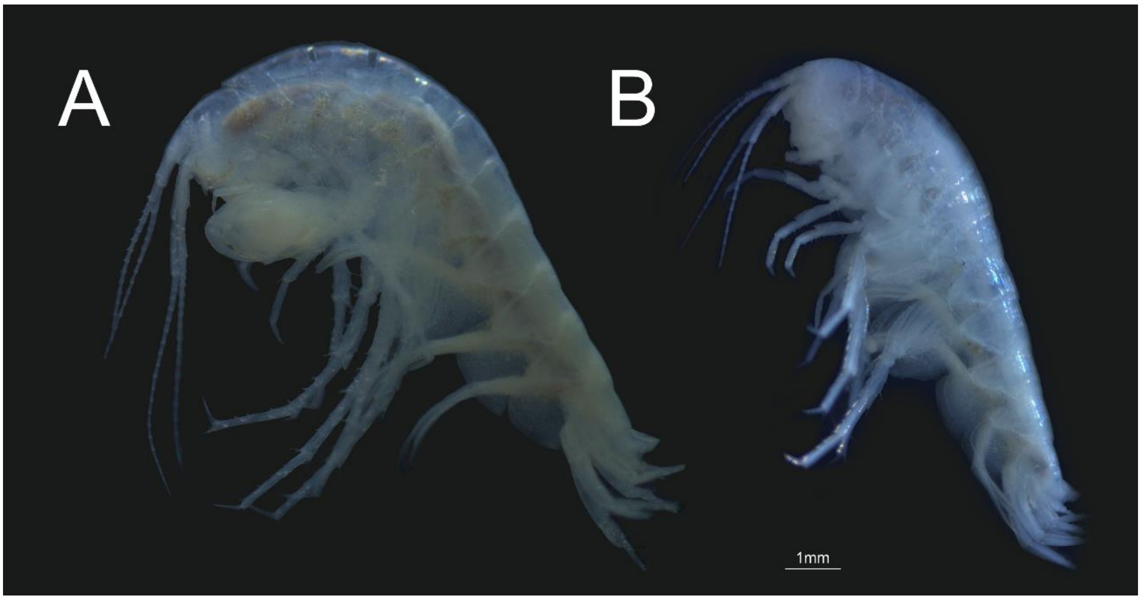

Figure 3: *Hyalella sp2* n. sp., Santana Cave, Iporanga municipality, state of São Paulo, Brazil. Holotype male, x.x mm (MNRJcarcino xxxxx) (**A**). Allotype female, x.x mm (MNRJcarcino xxxxx) (**B**). Scale bars = 1 mm.

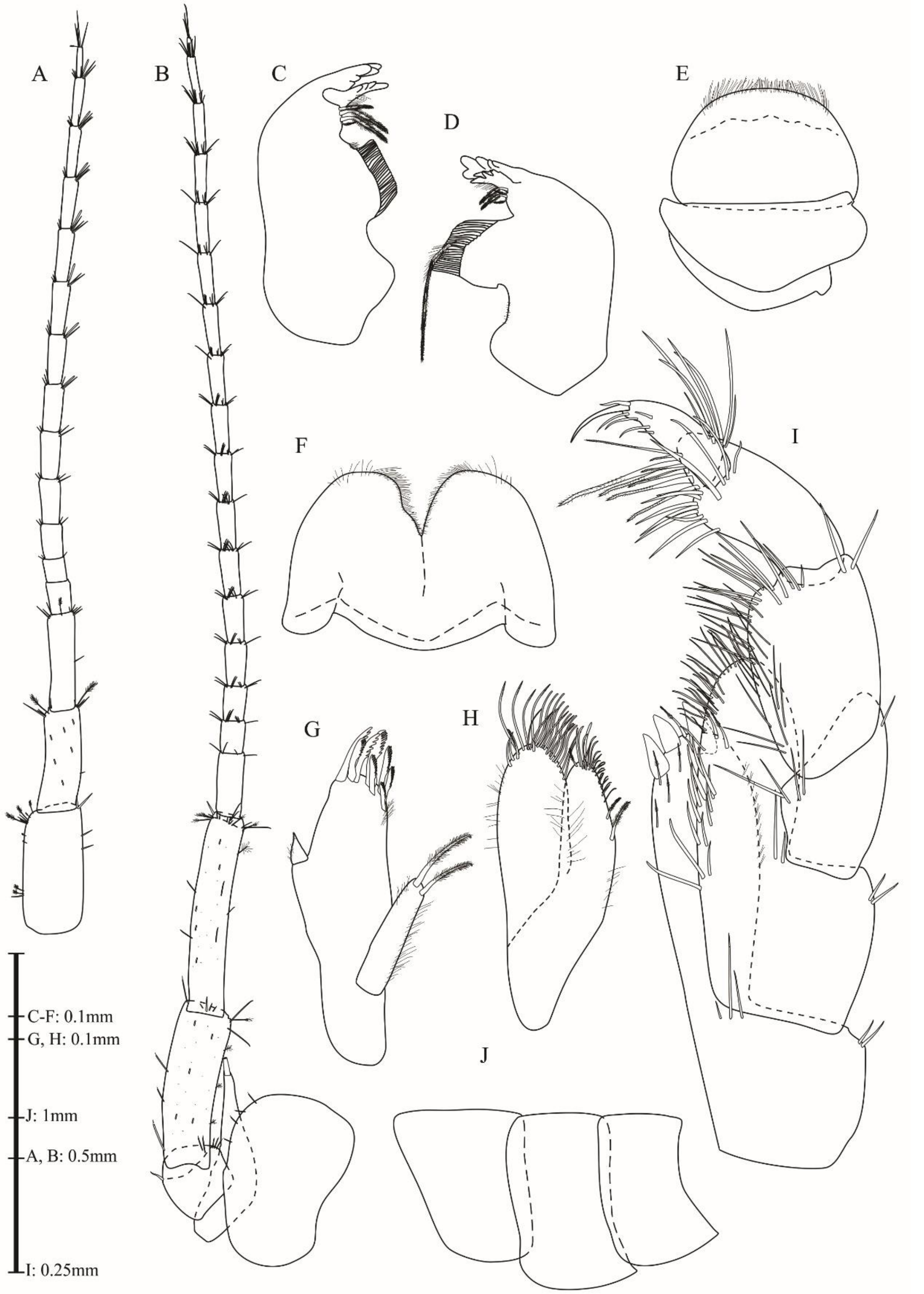

Figure 4: *Hyalella sp2* n. sp., Santana Cave, Iporanga municipality, state of São Paulo, Brazil. Paratype male, 5.29 mm; antenna 1 (**A**); antenna 2 (**B**); left mandible (**C**); right mandible (**D**); upper lip (**E**); lower lip (**F**); maxilla 1 (**G**); maxilla 2 (**H**); maxilliped (**I**); epimeral plates (**J**). Scale bars: A and B = 0.5 mm; C–H = 0.1 mm; I = 0.25 mm; J = 1 mm.

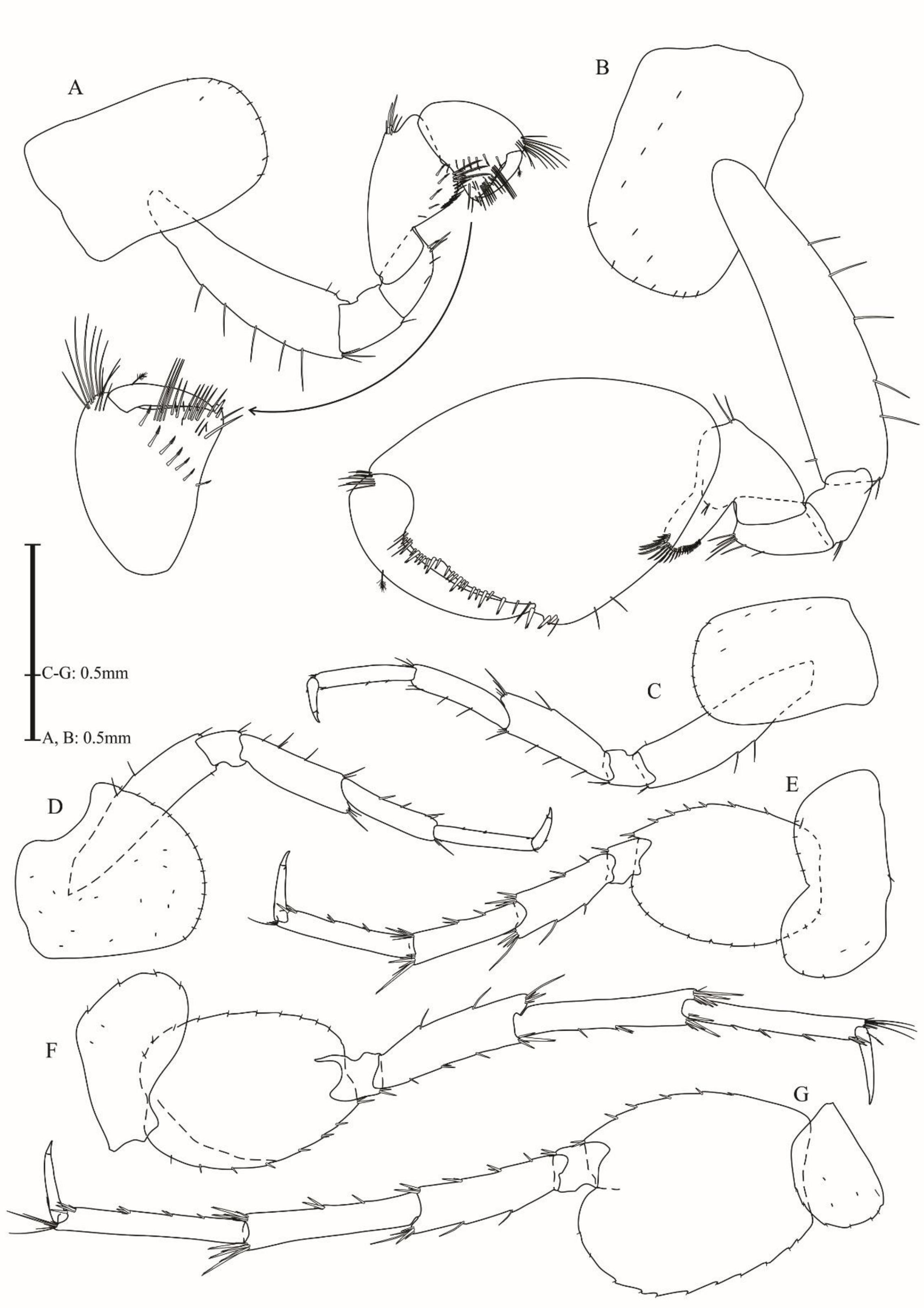

Figure 5. *Hyalella sp2* n. sp., Santana Cave, Iporanga municipality, state of São Paulo, Brazil. Paratype male, 5.29 mm; gnathopod 1 (**A**); gnathopod 2 (**B**); pereopod 3 (**C**); pereopod 4 (**D**); pereopod 5 (**E**); pereopod 6 (**F**); pereopod 7 (**G**). Scale bars = 0.5 mm.

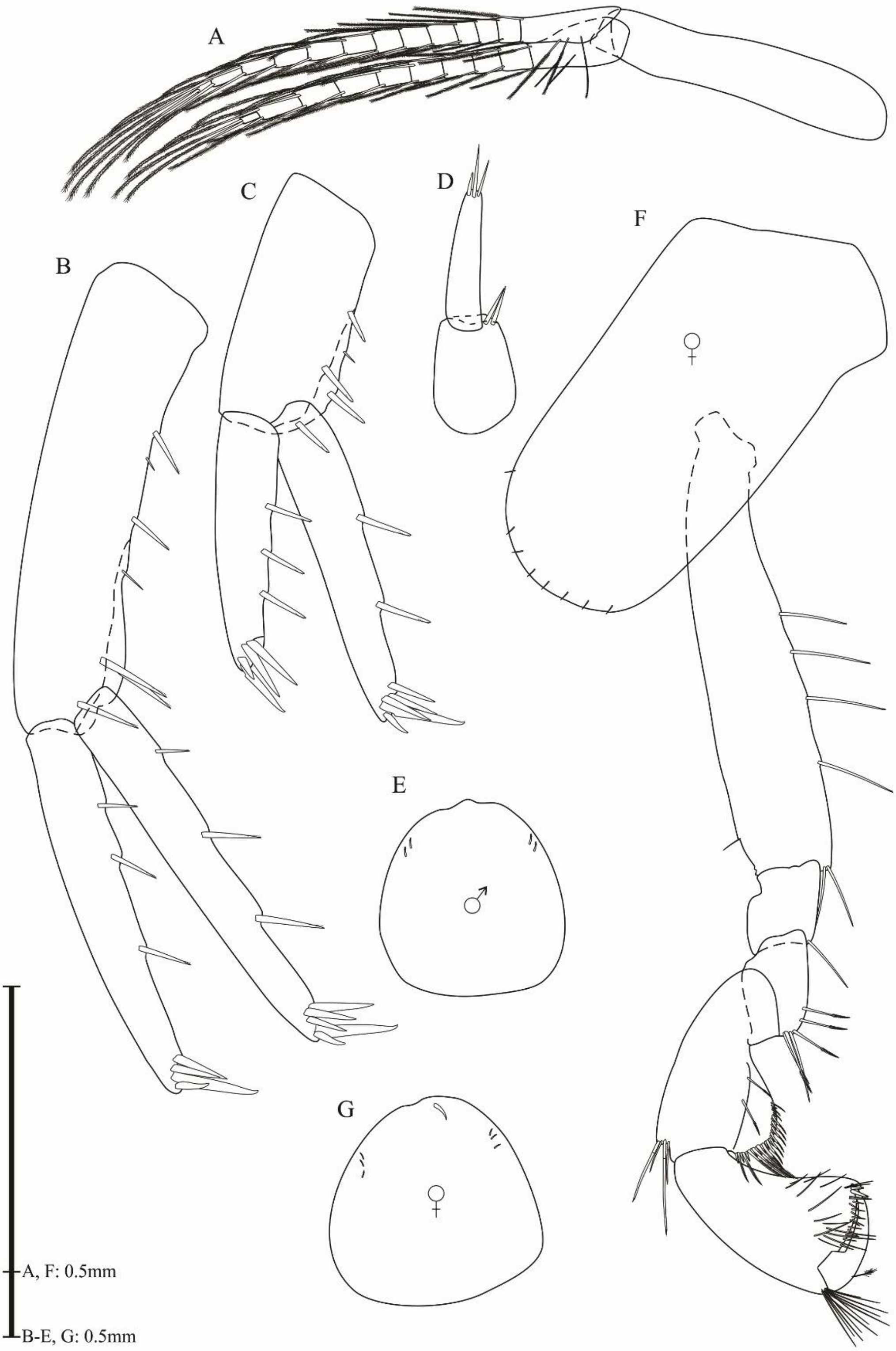

Figure 6. *Hyalella sp2* n. sp., Santana Cave, Iporanga municipality, state of São Paulo, Brazil. Paratype male, 5.29 mm, paratype female, 6.01 mm; pleopod (**A**); uropod 1 (**B**); uropod 2 (**C**); uropod 3 (**D**); male telson (**E**); female gnathopod 2 (**F**); female telson (**G**). Scale bars = 0.5 mm.

Figure 7: *Hyalella sp3* n. sp., Santana Cave, Iporanga municipality, state of São Paulo, Brazil. Holotype male, x.x mm (MNRJcarcino xxxxx) (**A**). Allotype female, x.x mm (MNRJcarcino xxxxx) (**B**). Scale bar = 2 mm.

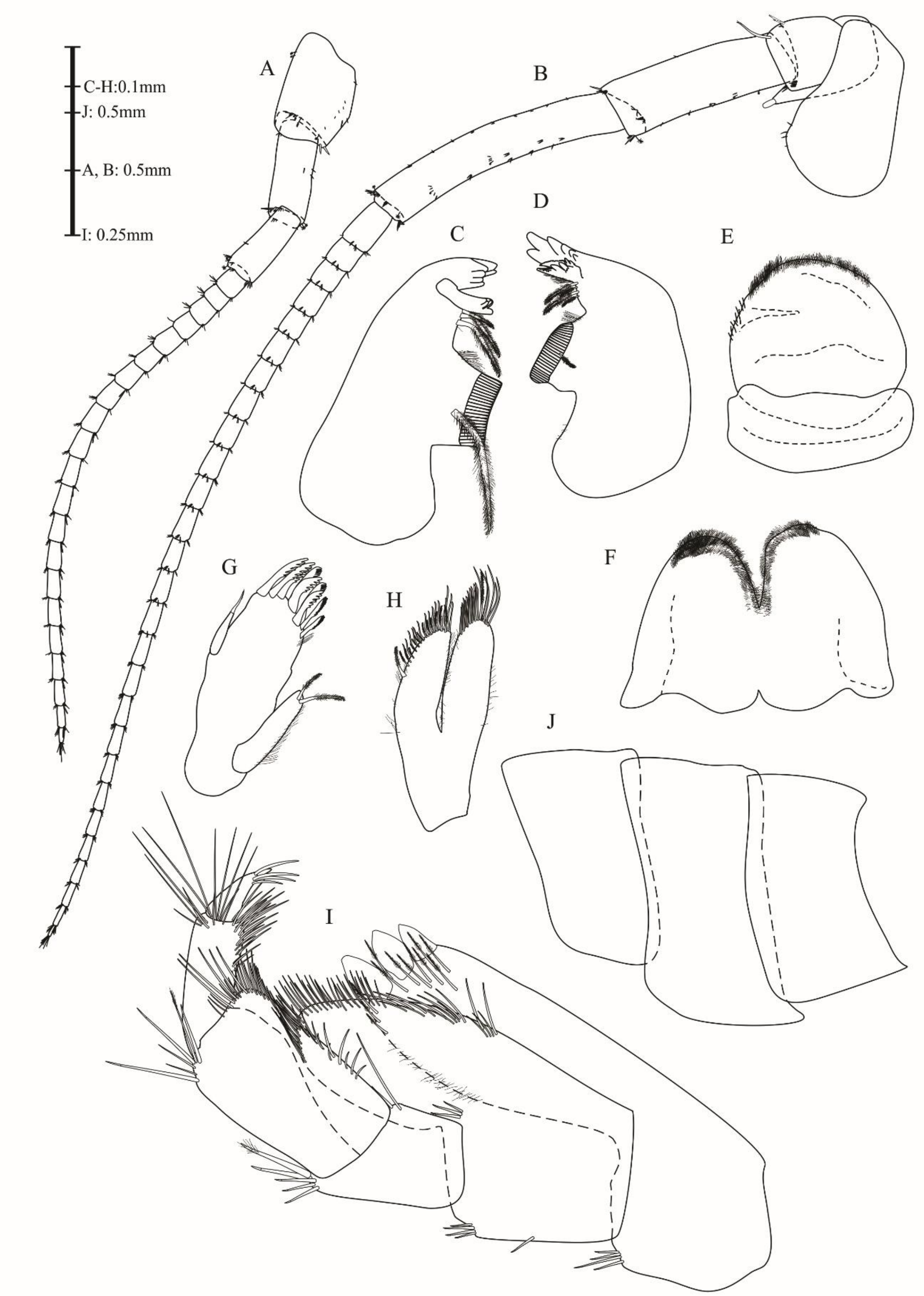

Figure 8: *Hyalella sp3* n. sp., Santana Cave, Iporanga municipality, state of São Paulo, Brazil. Paratype male, 9.69 mm; antenna 1 (**A**); antenna 2 (**B**); left mandible (**C**); right mandible (**D**); upper lip (**E**); lower lip (**F**); maxilla 1 (**G**); maxilla 2 (**H**); maxilliped (**I**); epimeral plates (**J**). Scale bars: A, B and J = 0.5 mm; C–H = 0.1 mm; I = 0.25 mm.

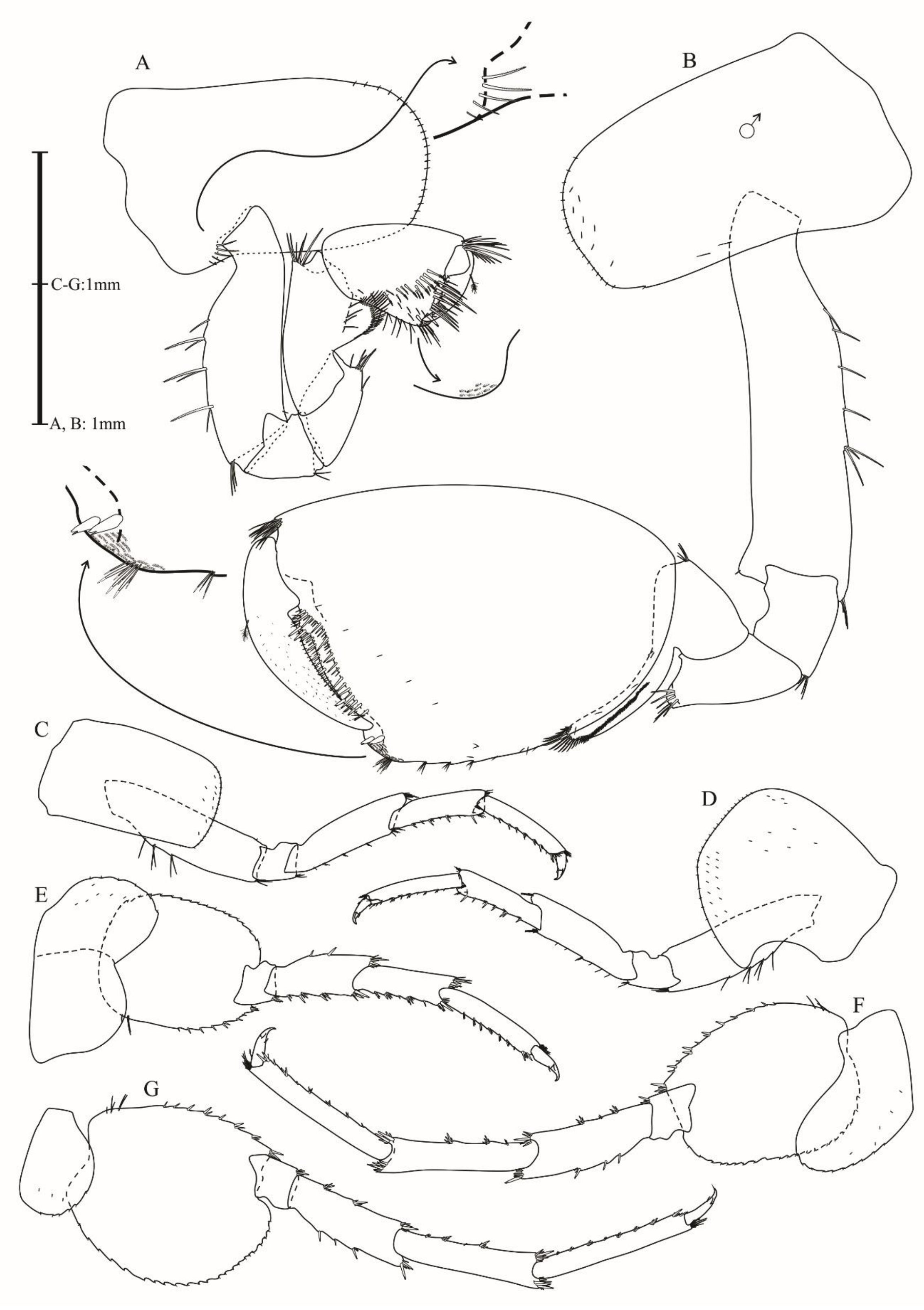

Figure 9. *Hyalella sp3* n. sp., Santana Cave, Iporanga municipality, state of São Paulo, Brazil. Paratype male, 9.69 mm; gnathopod 1 (**A**); gnathopod 2 (**B**); pereopod 3 (**C**); pereopod 4 (**D**); pereopod 5 (**E**); pereopod 6 (**F**); pereopod 7 (**G**). Scale bars = 1 mm.

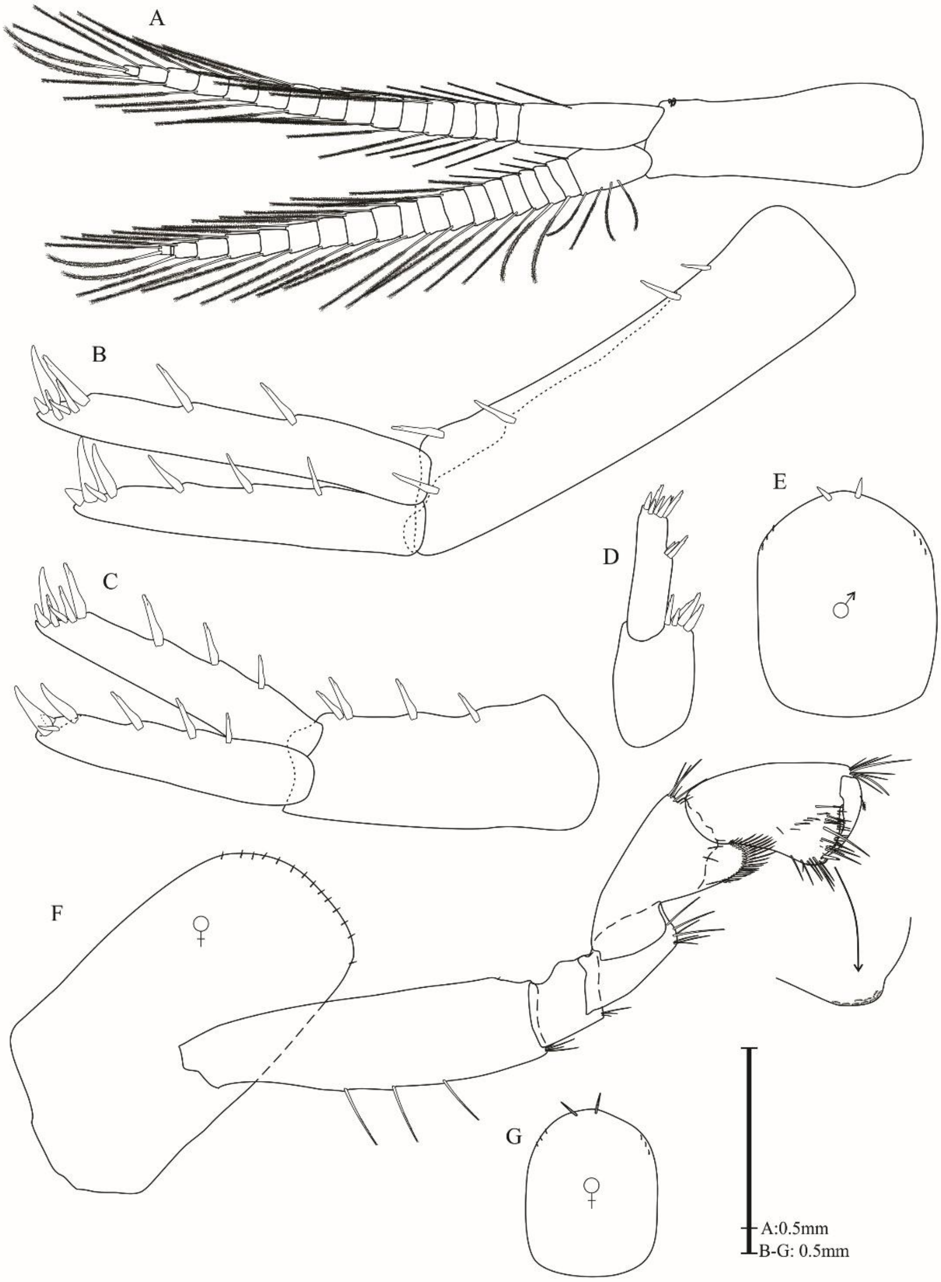

Figure 10. *Hyalella sp3* n. sp., Santana Cave, Iporanga municipality, state of São Paulo, Brazil. Paratype male, 9.69 mm, paratype female, 6.01 mm; pleopod (**A**); uropod 1 (**B**); uropod 2 (**C**); uropod 3 (**D**); male telson (**E**); female gnathopod 2 (**F**); female telson (**G**). Scale bars = 0.5 mm.

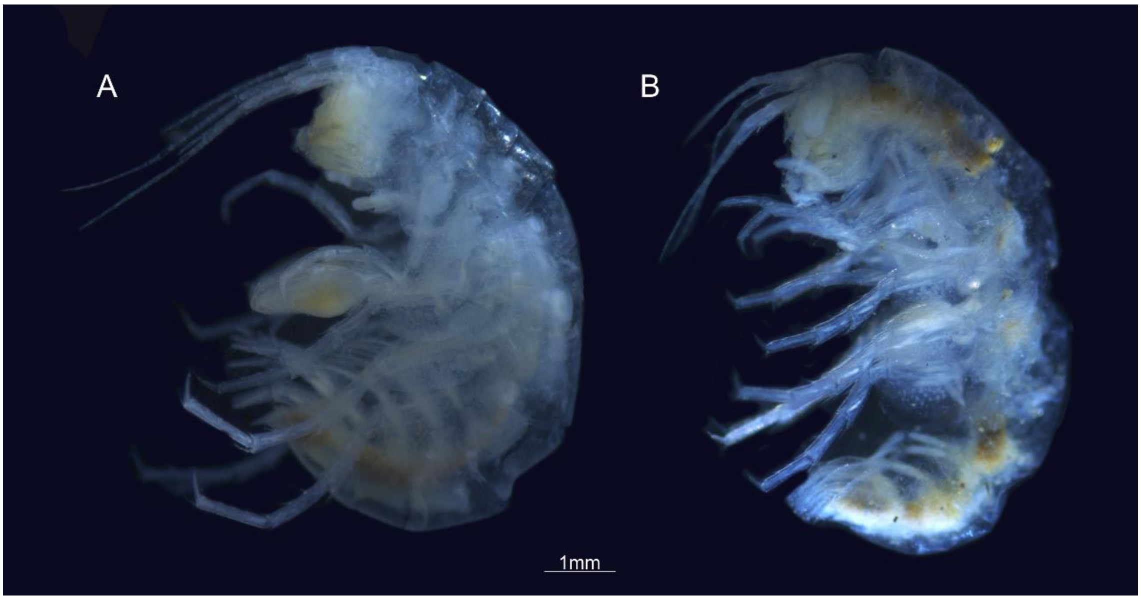

Figure 11: *Hyalella caeca* Pereira, 1989, “Gruta Tobias de Baixo” Cave, Iporanga municipality, state of São Paulo, Brazil. Holotype male, x.x mm (MNRJcarcino xxxxx) (**A**). Allotype female, x.x mm (MNRJcarcino xxxxx) (**B**). Scale bar = 1 mm.

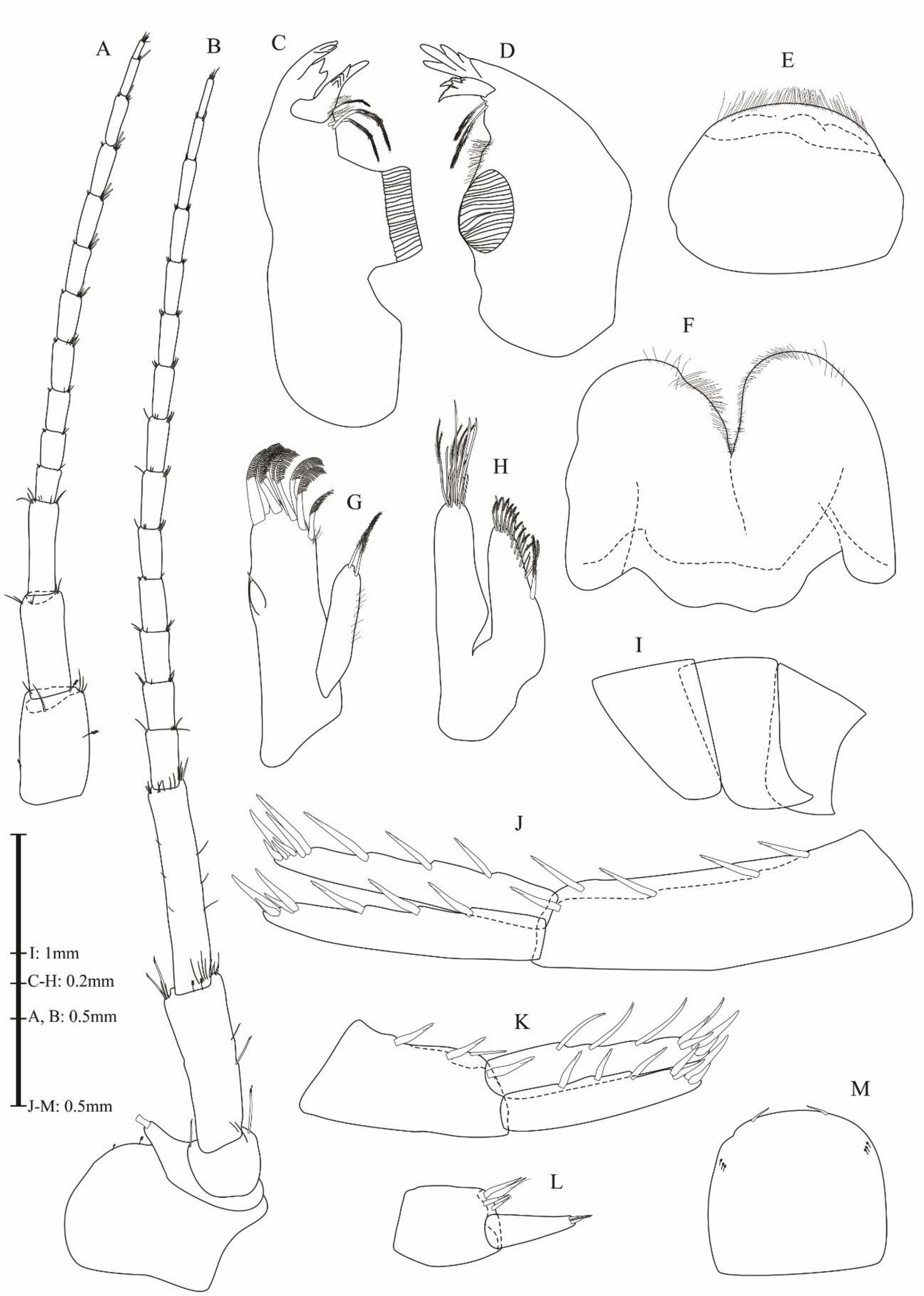

Figure 12: *Hyalella caeca* Pereira, 1989, “Gruta Tobias de Baixo” Cave, Iporanga municipality, state of São Paulo, Brazil. Paratype male, 7.00 mm; antenna 1 (**A**); antenna 2 (**B**); left mandible (**C**); right mandible (**D**); upper lip (**E**); lower lip (**F**); maxilla 1 (**G**); maxilla 2 (**H**); epimeral plates (**I**); uropod 1 (**J**); uropod 2 (**K**); uropod 3 (**L**); telson (**N**). Scale bars: A, B, J–M = 0.5 mm; C–H = 0.2 mm; I = 1 mm.

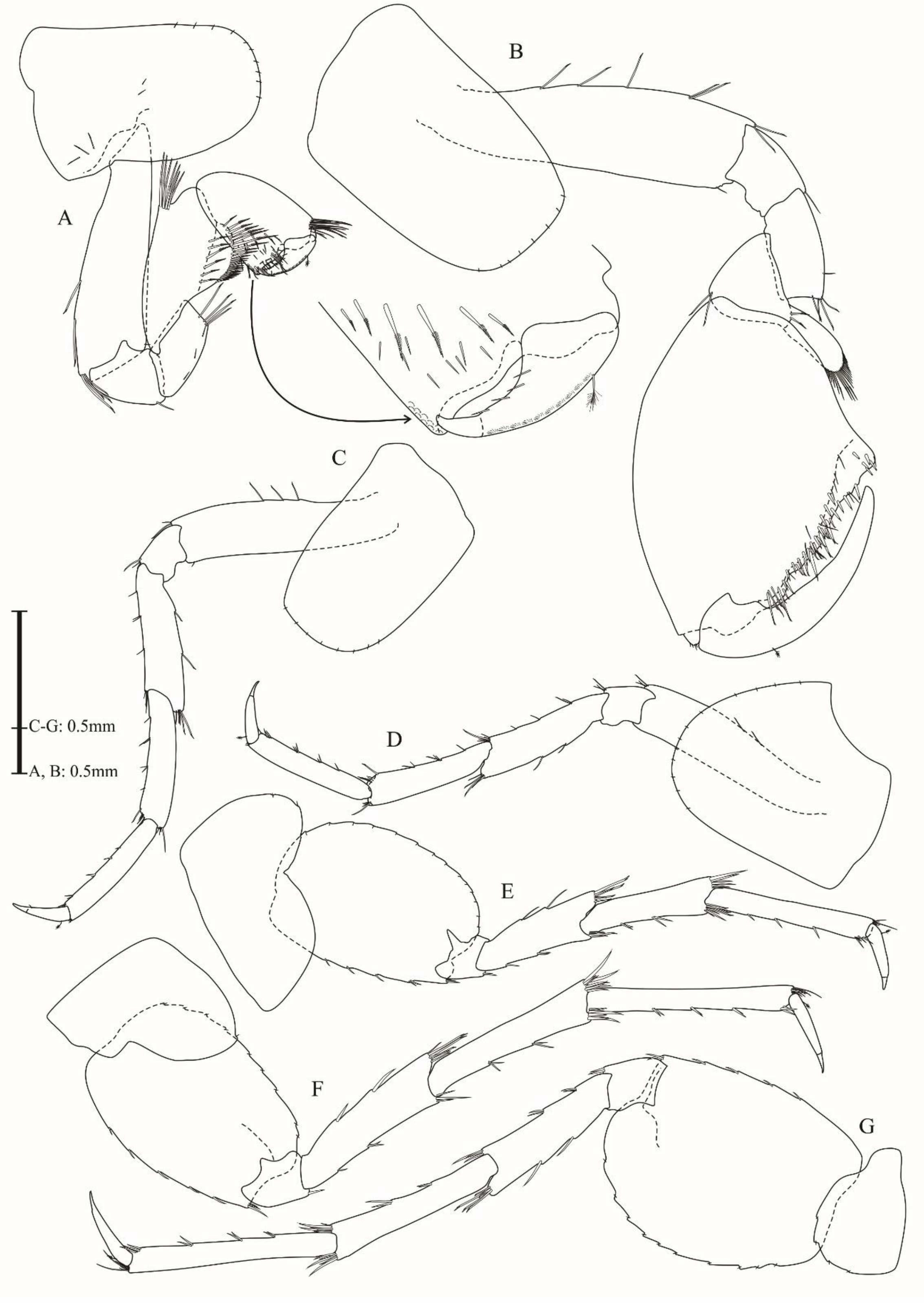

Figure 13. *Hyalella caeca* Pereira, 1989, “Gruta Tobias de Baixo” Cave, Iporanga municipality, state of São Paulo, Brazil. Paratype male, 7.00 mm; gnathopod 1 (**A**), gnathopod 2 (**B**), pereopod 3 (**C**), pereopod 4 (**D**), pereopod 5 (**E**), pereopod 6 (**F**), pereopod 7 (**G**). Scale bars = 0.5 mm.

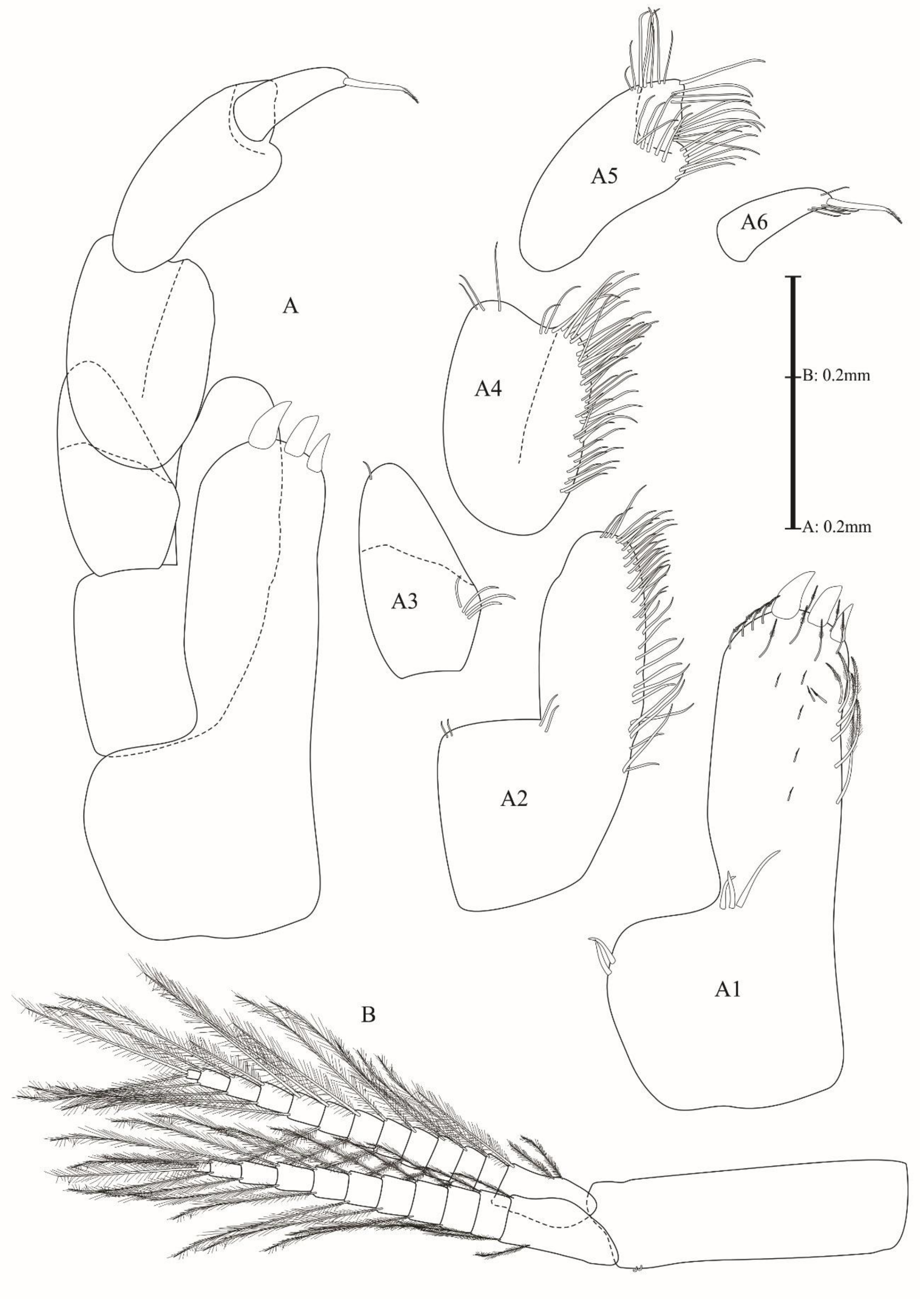

Figure 14. *Hyalella caeca* Pereira, 1989, "Gruta Tobias de Baixo" Cave, Iporanga municipality, state of São Paulo, Brazil. Paratype male, 7.00 mm, paratype female, 6.01 mm; maxilliped (**A**) – inner plate (**A1**), outer plate (**A2**), palp articles 1–4 (**A3–6**); pleopod (**B**). Scale bars = 0.2 mm.

Tables:

Table 1: Morphological characteristics of hypogean (subterranean) species of *Hyalella* from Brazil. Legend: [h]: hypothelminorheic; *: new species, under description; [a]: superficial, but with "troglomorphies"; Y/N/R: yes/no/reduced; **bold**: equal to *H. sp2* n. sp.; *italic*: equal to *H. caeca* Pereira, 1989; A1/2: antenna 1/2; M1: maxilla 1; OP: outer plate; G1/2: gnathopod 1/2; CS: comb scales; PP: polygonal pattern; U1: uropod 1; BL: body length; DPC: disto-posterior corner.

Species	Eyes	A1xA2 relative length	A2 relative size	M1 OP serrate setae	M1 relative palp length	G1 CS/PP propodus	G2 CS/PP propodus	U1 curved seta	G2 palm excavation	Coxal gills	Sternal gills
H. sp2 n. sp.	***No***	***A1<A2***	**>1/2 BL**	**8**	***<1/2***	**No**	***No***	***No***	***Yes***	***2-6***	***2-7***
H. caeca	***No***	***A1<A2***	*<1/2 BL*	*9*	***<1/2***	*CS DPC*	***No***	***No***	***Yes***	***2-6***	***2-7***
*H. sp*4*	Yes	***A1<A2***	≅1/2	10	***<1/2***	*CS DPC*	CS DPC	***No***	***Yes***	***2-6***	***2-7***
H. sp5*	Yes	***A1<A2***	≅1/2	*9*	***<1/2***	PP DPC & CS AM	PP DPC	***No***	***Yes***	***2-6***	***2-7***
H. epikarstica	***No***	A1>A2	*<1/2 BL*	*9*	***<1/2***	**No**	***No***	Yes	No	**2-6**	3-7
H. formosa	***No***	A1>A2	*<1/2 BL*	*9*	***<1/2***	**No**	***No***	Yes	No	**2-6**	***2-7***
H. imbya[h]	***No***	A1>A2	*<1/2 BL*	***8-9***	***<1/2***	**No**	***No***	Yes	***Yes***	**2-6**	3-7
H. spelaea	Y/N/R	***A1<A2***	**>1/2 BL**	*9*	≅1/2	*CS DPC*	***No***	***No***	***Yes***	2-7	2-6
*H. temimina**	Yes	***A1<A2***	*<1/2 BL*	*9*	>1/2	PP DPC	PP DPC	***No***	***Yes***	***2-6***	3-7
H. troglofugia[a]	***No***	***A1<A2***	*<1/2 BL*	**8**	***<1/2***	*CS DPC*	CS DPC	***No***	No	2-7	***2-7***
H. veredae	Y/N/R	***A1<A2***	*<1/2 BL*	***8-9***	***<1/2***	*CS DPC*	CS DPC	Yes	***Yes***	**2-6**	***2-7***

Table 2: Morphological characteristics of epigean (superficial) species of *Hyalella* from Brazil. Legend: A2: antenna 2; M1: maxilla 1; OP: outer plate; G1/2: gnathopod 1/2; CS: comb scales; PP: polygonal pattern; BL: body length; DPC: disto-posterior corner; AM: anterior margin; **bold**: equal to *H. sp3* n. sp.; ??: not specified or unidentified; *: new species, under description.

Species	A2 relative size	M1 OP serrate setae	M1 relative palp length	G1 CS/PP propodus	G2 CS/PP propodus	G2 palm excavation	Accumination on epimeral plates	Coxal gills	Sternal gills
H. sp3 n. sp.	**>1/2 BL**	**9**	**≅1/2**	**PP DPC**	**PP DPC**	**Yes**	**2.3**	**2-6**	**2-7**
H. bala	<1/2 BL	8	≅1/2	No	No	Yes	2	2-6	2-7
H. sp4*	≅1/2	10	<1/2	CS DPC & AM	CS DPC	Yes	1.2.3	**2-6**	**2-7**
H. dielaii	<1/2 BL	9	<1/2	No	No	No	2.3	3-6	2-7
H. gracilicornis	**>1/2 BL**	**9**	**≅1/2**	CS DPC & AM	CS DPC	No	1.2.3	**2-6**	**2-7**
H. longiprodus	**>1/2 BL**	**9**	<1/2	CS DPC & AM	CS DPC	**Yes**	1.2.3	3-6	**2-7**
H. longistila	**>1/2 BL**	**9**	<1/2	CS DPC & AM	CS DPC	**Yes**	1.2.3	**2-6**	**2-7**
H. minensis	<1/2 BL	7	<1/2	CS DPC	No	Yes	??	2-7	2-7
H. montana	<1/2 BL	8	**≅1/2**	No	No	No	1.2.3	2-6	3-7
*H. bocaina**	<1/2 BL	**9**	**≅1/2**	**PP DPC** & AM	**PP DPC**	No	**2.3**	2-7	3-7
H. virgineae	≅1/2 BL	**9**	≅1/4	**PP DPC**	No	Yes	2.3	2-6	2-7
H. warmingi	**>1/2 BL**	**9**	<1/2	No	No	**Yes**	1.2.3	**2-6**	**2-7**

4. Capítulo 4

Artigo: Two new species of *Hyalella* Smith, 1874 (Amphipoda: Hyalellidae) from Southeast Brazil, with a pictorial key to the hypogean species of the genus

Artigo submetido para o periódico Zootaxa

Two new species of *Hyalella* Smith, 1874 (Amphipoda: Hyalellidae) from Southeast Brazil, with a pictorial key to the hypogean species of the genus

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Abstract

The amphipod genus *Hyalella* can be found throughout the freshwater environments in the American continent. There are both superficial (epigean) and subterranean (hypogean) *Hyalella* species, with most of its underground populations found in the Brazilian territory. Here we describe two new cave species of the genus for Southeast Brazil and present a pictorial key to identify the subterranean Brazilian *Hyalella*.

Keywords: freshwater amphipods; morphology; subterranean; taxonomy.

Introduction

Hyalella Smith, 1874 is the only genus of the Hyalellidae Bulyčheva, 1957 family (Rogers *et al.* 2020). Unlike many other amphipod families, hyalellids present a uniramous uropod 3 and mandibles without palp (Rogers *et al.* 2020). The Brazilian territory holds more than two thirds of the genus known richness, with species found throughout the Southeastern and Southern states (Penoni *et al.*, 2021; Limberger *et al.*, 2022; Rangel *et al.*, 2022).

There are six hypogean *Hyalella* species described to Brazil to this day: *Hyalella caeca* Pereira, 1989, found at Gruta Tobias (or Gruta Tobias de Baixo, as indicated at the description paper), Iporanga municipality, São Paulo state; *H. spelaea* Bueno & Cardoso, 2011, found at Gruta do Toca, Itirapina municipality, São Paulo state (Cardoso *et al.*, 2011); *H. formosa* Cardoso & Araújo, 2014, found at Andorinhas Cave, Ponta Grossa municipality, Paraná state (Araújo *et al.*, 2014); *H. imbya* Rodrigues & Bueno, 2012, found at Roque González municipality, Rio Grande do Sul state (Rodrigues *et al.*, 2012); *H. epikarstica* Rodrigues, Bueno & Ferreira, 2014, found at Areias de Cima cave, Iporanga municipality, São Paulo state (Rodrigues *et al.*, 2014); and *H. veredae* Cardoso & Bueno, 2014, found at Vereda da Palha cave, Presidente Olegário municipality, Minas Gerais state (Araújo *et al.*, 2014). All but one are found in caves – *H. imbya* is hypotelminorheic (Rodrigues *et al.*, 2012). There is one species with troglomorphic characteristics, *H. troglofugia* Bastos-Pereira, Oliveira & Ferreira, 2018, only this one is epigean (Bastos-Pereira *et al.*, 2018).

Here we describe two new cave species of the genus *Hyalella*, one found at Parque Estadual Intervales (PEI/SP), Iporanga municipality, and the other found at Parque Estadual da Caverna do Diabo, Eldorado municipality, both on the state of São Paulo. These species, although subterranean, are not blind and do not present any evident signs of troglomorphisms. We also present a pictorial key to identifying subterranean *Hyalella*, both those with evident troglomorphisms and those with no apparent specific adaptations to life underground.

Materials and methods

Study area

Parque Estadual Intervales has more than 60 registered caves divided in two groups – the Bocaina/Lajeado grouping and the Headquarters grouping (São Paulo, 2010). The Jane Mansfield Cave (Fig. 1) is located at the Bocaina/Lajeado grouping; it is a touristic cave characterized by a single main corridor, few bifurcations (Rocha & Galvani, 2013), and adorned with stalagmites, stalactites, and columns (São Paulo, 2010). The cave is formed by a small superior entrance at the river resurgence, with a development of ca. 420 m. The main water body is characterized by a rocky substrate with some patchy areas of sandy and gravel substrate. The water current is moderate with most with prevailing lentic and shallow parts.

Parque Estadual da Caverna do Diabo (PECD) (Fig. 1B; Fig. 2A) was built around "Gruta da Tapagem" Cave, most popularly known as "Caverna do Diabo", the largest touristic cave of the state of São Paulo (São Paulo, 2010). Both PECD and PEI are characterized by the Atlantic Rainforest biome and are on the geographical limits of the "Vale do Ribeira", a region of great biotic importance, with an elevated degree of endemism (São Paulo, 2010).

The Gruta da Tapagem Cave is a partially touristic cave that receives water from the epigean stream "Córrego da Tapagem" (or Rio das Ostras). It is one of the largest caves in the state of São Paulo, with 8 km of mapped passageways (GBPE, pers. comm., 2022). On the outside of the cave, the river is mostly of rapid shallow water; the subterranean drainage has many soft-bottomed pools, some deep (> 2.5 m of depth) intercalated by rapid waters. There are important human impacts on the subterranean drainage due to the tourism, such as dams and artificial lights. *Hyalella* specimens were found on the river margins associated to the aquatic vegetation in the epigean stream, and on small soft-bottomed pools in the subterranean stretches.

Hyalella collection

Collections at Jane Mansfield Cave happened under collection authorization SISBIO/ICMBio (20165) and Fundação Florestal-SP, as part of the speleological management plan. *Hyalella* was collected with the aid of small hand nets and brushes and preserved in identified tubes filled with 70% ethyl alcohol.

Collections at Gruta da Tapagem Cave happened under the collection authorization SISBIO/ICMBio (75338) and the Instituto Florestal – Secretaria de Infraestrutura e Meio Ambiente-SP (004738/2020-15). For collections at PECD, *Hyalella* specimens were sampled in different locations, both inside – on the touristic and non-touristic paths – and outside of the cave. The collection sites inside of the cave were lentic pools where the animals were swimming freely; while outside, the water current was stronger, and the collected animals were attached to plant roots. *Hyalella* was collected using plastic pipettes directly for the lentic pools and with the aid of hand nets for the lotic sites. Couples in pre-copulatory behavior and visibly ovigerous females were individualized. Most specimens were placed on identified plastic tubes with ethanol 70%, and at least two of each collection site was placed at identified glass tubes with ethanol 100%.

Identification and species description

For description procedures, selected individuals from both populations were dyed in “Red Congo” dye for at least 24h. Slide preparation and drawing procedures followed Reis *et al.* 2020. Nomenclatural description followed Rogers *et al.* 2020 and Zimmer *et al.* 2009.

Type material was deposited in the crustacean collection of Museu Nacional do Rio de Janeiro (MNRJcarcino), Rio de Janeiro, Brazil, Coleção de Crustáceos da Universidade Federal de Lavras (CCUFLA) and at the Zoological Collection of the Laboratório de Estudos Subterrâneos, Universidade Federal de São Carlos (LES).

Production of a pictorial key for species identification

To produce the pictorial key for identification of Brazilian hypogean *Hyalella* species, we first organized a table with all the main morphological characteristics used for *Hyalella* identification of species. We then created drawings using CorelDRAW X7 to illustrate these characteristics. Drawings were based on the original descriptions of species and on photographs of appendages mounted on semi-permanent slides. The pictorial key was assembled using CorelDRAW X7.

TAXONOMY

Order Amphipoda Latreille, 1816
Suborder Senticaudata Lowry & Myers, 2013
Family Hyalellidae Bulyčheva, 1957
Genus *Hyalella* Smith, 1874
Hyalella sp4 n. sp. Penoni & Bueno (Figs. 3–6)

Type material: Holotype male (Fig. 3A), body length = 6.84 mm, head length = 0.7 mm, Caverna do Diabo, Parque Estadual da Caverna do Diabo, Eldorado municipality, São Paulo state, Brazil (24°42'47" S; 48°25'6" W), MNRJcarcino XXXXX, Feb. 2022, Bichuette, M.E., Penoni, L.R., Zepon, T. coll.; allotype female (Fig. 3B), body length = 5.06 mm, head length = 0.68 mm, MNRJcarcino XXXX; paratype 36 hole individuals CCUFLA 464, one male in slides CCUFLA 462 and one female in slides CCUFLA 463 (same collection data as holotype).

Diagnosis: Body surface smooth. Epimeron 2 and 3 postero-distal margin sub-acute. Eyes present, normal. Antenna 1 0.6× the length of Antenna 2, almost twice as long as the ramus of Antenna 2. Antenna 2 subequal to half the body length. Maxilla 1 with 10 serrate setae on the outer plate. Gnathopod 1 of male and female identical, with comb scales on the ischium and merus distal margins, propodus disto-posterior corner and anterior margin, and on the dactylus. Gnathopod 2 propodus ovate, palm with and excavation, slope oblique, comb scales on the disto-posterior corner and anterior margin, and on the dactylus. Pereopods 3–5 subequal in size. Uropod 3 longer than the peduncles of uropods 1 and 2, and longer than the telson, peduncle wider and shorter than the ramus. Coxal gills on segments 2–6. Sternal gills on segments 2–7.

Male: Mean body length 6.58 ± 0.36 mm (N = 3) (6.17 – 6.84 mm); mean head length 0.60 ± 0.03 mm (N = 3) (0.58 – 0.64 mm). Body surface smooth. Epimeral plates 2 and 3 sub-acute (Fig. XJ). Coxae 1–3 similar, subequal in size, with some scattered microtrichs on coxae 2 and 3; coxa 4 as wide as long, excavated posteriorly. Eyes big and round.

Antenna 1 (Fig. 4A) 0.4× the body length, 0.6× the length of Antenna 2, 1.9× longer than the peduncle of Antenna 2 and 0.8× the length of the flagellum of Antenna 2. Flagellum with 13 articles; aesthetascs occurring distally on flagellum after article 6.

Antenna 2 (Fig. 4B) almost half the body length (0.46× the body length); peduncle slender, 2.3× the length of the head; flagellum with 17–21 articles, 2.5× longer than the peduncle.

Mandible without palp; incisor toothed; left (Fig. 4C) *lacinia mobilis* with 5 teeth, setae row with 6 pappose setae (3 stronger) and a few setules, accessory seta on the molar process; right mandible (Fig. 4D) with 2 pappose setae and some setules; molar process cylindrical, accessory seta present.

Upper lip (Fig. 4E) margin rounded; distal border covered by setules. Lower lip (Fig. 4F) outer lobes rounded, distally notched, covered by setules apically.

Maxilla 1 (Fig. 4G) inner plate slender, with 2 apical pappose setae and a few lateral setules. Outer plate with 10 serrate setae apically. Palp short, uniarticulate, not reaching half the distance between base of the palp and base of the setae on the outer plate, 1 short cuspidate seta apically and setules laterally.

Maxilla 2 (Fig. 4H) inner plate shorter and wider than outer plate, with 1 strong pappose setae distally and paired simple and papposerrate seta disto-apically, few setules scattered on the margins; outer plate with several long simple setae disto-apically, setules on the inner margin.

Maxilliped (Fig. 4I) without comb scales; inner plate 1.4× longer than outer plate, with 3 large cuspidate setae apically, pappose setae disto-apically, setules on the inner face, 3 simple setae on the outer margin; outer plate with several long simple setae on the inner margin, 3 simple seta on the inner face, and up to 2 simple setae on the outer margin; palp 1.3× longer than plate, with 4 articles; article 1 outer margin with 2 simple setae inner margin with 4 simple setae (2 longer); article 2 inner and disto-inner margins with several simple setae disto-outer margin with 4 simple setae; article 3 inner margin and outer margins with long simple setae, papposerrate setae on the distal and outer margins; article 4 (dactylus) unguiform with distal serrate nail present, with up to 6 simple setae.

Gnathopod 1 (Fig. 5A) subchelate, identical on the male and the female; coxal plate 1.7× wider than long, with simple setae on the anterior margin, 1 pappose or papposerrate setae on the posterior inner face, close to the attachment of the basis; basis with 1 simple seta with accessory seta on the posterior margin, 1 simple seta and 2 serrate setae on the disto-posterior margin and 1 short simple seta on the disto-anterior margin; ischium with 2 apical simple setae, comb scales present; merus with 4 simple setae with accessory seta and comb scales on distal margin; carpus longer than wide, with pappose setae on the lateral distal lobe, 4 papposerrate setae on inner face and up to 9 serrate and papposerrate setae distally; propodus 1.5× longer than wide, hammer shaped, 2 simple setae on the posterior margin, with comb scales on the disto-posterior and disto-anterior margins and some long and short simple setae on disto-anterior margin; palm slope transverse, with long and short simple setae; 7 long pappose setae and some smaller simple setae on inner face; dactylus claw-like, small simple setae on the proximal margin, polygonal pattern on the distal margin, with a plumose seta distally.

Gnathopod 2 (Fig. 5B) subchelate; coxal plate 1.7× wider than long, with simple setae on the margin and some scattered microtrichs on the inner face; basis with 1 simple seta with accessory seta on posterior margin, 1 short simple setae on anterior margin, and 2 simple setae disto-posteriorly; ischium with 3 simple setae and comb scales disto-posteriorly; merus with 1 simple seta and 5 serrate setae on distal margin, comb scales present; carpus lobe slim, produced between merus and propodus, posterior margin with several pappose setae, disto-anterior margin with 3 serrate setae; propodus ovate, slope oblique, posterior margin with 3 groups of 1 to 2 simple setae and comb scales, palm with wide posterior excavation, with short cuspidate setae with accessory setae and simple setae, anterior margin with several simple setae; dactylus claw-like, congruent with palm, with small simple setae on the proximal margin and a plumose seta distally.

Pereopods 3–7 (Fig. 5C–G) simple. Coxae 3–7 with simple setae distally and microtrichs (except 7) on inner face; coxa 3 similar to 1 and 2; coxa 4 as long as wide, excavated posteriorly; coxa 5 posterior lobe as long as anterior lobe; coxa 6 anterior lobe reduced; coxa 7 reduced. Pereopods 3, 4 and 5 subequal in size, P4 slightly smaller; pereopods 3 and 4 setation similar, with 3 or 4 groups of 1 or 2 simple setae on the posterior margin of the merus, and 3 to 5 groups of 1 to 6 cuspidate setae with accessory seta and simple setae on the posterior margins of carpus and propodus, dactylus with a plumose seta; pereopod 3 basis with up to 3 simple setae on the posterior margin and pereopod 4 basis with a serrate seta on the posterior margin. Pereopods 5 to 7 setation similar, with 4 to 6 groups of 1 to 3 cuspidate setae with accessory seta on the anterior margin of the basis, 2 to 3 groups of 2 to 4 cuspidate setae with accessory seta and simple seta on the anterior margins of the merus, carpus and propodus, 1 or 2 cuspidate setae with accessory seta on the posterior margin of the merus and a plumose seta on the dactylus. Pereopod 6 subequal in length to P7, ~1.4× longer than P3–P5.

Pleopods (Fig. 6A) peduncle half the length of the rami, 2 coupling spines; both rami with several long plumose setae.

Uropod 1 (Fig. 6B) peduncle 1.1× longer than inner ramus and 1.3× longer than the outer ramus, with up to 4 cuspidate setae with accessory seta an 2 small simple setae disto-laterally; inner ramus 1.1× longer than the outer ramus, with 2 cuspidate setae with accessory seta on dorsal margin and 7 cuspidate setae (2 longer, 5 shorter) apically, without curved seta; outer ramus with 3 cuspidate setae on dorsal margin and 4 cuspidate setae (1 longer, 3 shorter) apically.

Uropod 2 (Fig. 6C) 0.7× the total length of Uropod 1, 1.3× longer than the peduncle of Uropod 1, peduncle subequal in length to the outer ramus, and 0.8× the length of the inner ramus, peduncle with 4 cuspidate setae with accessory seta disto-laterally; inner ramus with 2 cuspidate setae with accessory seta on dorsal margin and 6 cuspidate setae (2 longer, 2 medium, and 2 shorter) apically; outer ramus with 3 cuspidate setae with accessory seta on dorsal margin and 4 cuspidate setae (1 longer, 3 shorter) apically.

Uropod 3 (Fig. 6D) 0.7× the length of the peduncle of uropod 1, 1.1× longer than the peduncle of uropod 2, 1.6× longer than the telson; peduncle with up to 7 cuspidate setae with accessory seta on outer distal margin and some scattered small simple setae on the inner face; inner ramus absent; outer ramus 1.3× longer than the peduncle, with 6 cuspidate setae apically.

Telson (Fig. 6E) entire, longer than wide, apically rounded and with 1 long simple seta apically and 1 short plumose seta on one of the sides.

Epimeron (Fig. 4J) 1 postero-distal margin rounded, 2 and 3 postero-distal margin sub-acute; epimeron 2 slightly longer than 3, epimeron 1 the smallest.

Coxal gills sac-like on pereonites 2-6. Sternal gills tubular on pereonites 2-7.

Female: (Fig. 6F–G) Mean body length 5.78 ± 0.62 mm (N = 3) (5.06 – 6.18 mm); mean head length 0.54 ± 0.04 mm (N = 3) (0.51 – 0.59 mm). Gnathopod 1 identical to the male's. Gnathopod 2 (Fig. 6F) similar in size and shape to Gnathopod 1,with only the propodus more elongated; basis posterior and distal margins and ischium posterior margin with serrate setae; merus disto-posterior margin with up to 6 serrate seta and one simple seta; carpus with 2 pappose seta on the inner face; propodus with a few scattered simple setae on the inner face; palm transverse, 0.4× the length of posterior margin, with a row of short simple setae, comb scales on the disto-posterior corner; dactylus claw-like with a distal plumose seta and comb scales. Telson (Fig. 6G) longer than wide, with 2 simple setae apically and 2 small plumose setae laterally.

Habitat: freshwater, troglophile – both epigean and hypogean. The specimens of *Hyalella sp4* n. sp. were found on small lentic pools inside "Caverna do Diabo", swimming freely on the water current, and also associated to vegetation and plant roots on the outside of the cave.

Conservation: H. sp4 n. sp. is classified as endangered (EN) based on IUCN subcategory B1 (< 5.000km^2 in area of occurrence extension) and D1 (< 250 mature individuals) (ICMBIO, 2013).

Remarks: We here present a pictorial key for identification of subterranean (troglobiotic and troglophilic) species of *Hyalella*. In addition to it, we highlight some of the main differences between *H. sp4* n. sp. and all other subterranean species of the genus: first, *H. sp4* is a troglophile species, with normal eyes, which easily differentiates it from *H. caeca*, *H. epikarstica*, *H. formosa*, *H. imbya*, *H. sp2* (Penoni et al., in press), and *H. troglofugia*; *H. spelaea* and *H. veredae* can have either regular or reduced eyes or yet, have no eyes. *Hyalella sp4* sp. n. has no curved seta on the inner ramus of the male's uropod 1, which differentiates it from *H. epikarstica*, *H. formosa*, *H. imbya* and *H. veredae*. When we use the relative length of antenna 2 to differentiate species, *H. sp4* n. sp. is similar only to *H. sp5* n. sp., with antenna 2 subequal to half the total body length; *H. sp2* and *H. spelaea* have antenna 2 longer than half the body length, and all the other species have shorter antennas 2. And finally, *H. sp4* n. sp. is the only species in the troglobiotic/troglophilic chategories with 10 serrate setae on the outer plate of maxilla 1; all the other have 8–9 serrate setae.

Hyalella sp5 n. sp. Penoni & Bueno (Fig. 7–9)

Type material: Holotype male (dissected, in slides), body length 6.91 mm; head length 0.61 m, Jane Mansfield cave, Parque Estadual Intervales, Iporanga municipality, São Paulo state, Brazil, (24°16'11" S; 48°26'41" W), MNRJcarcino xxxxx, March 2009, M.E. Bichuette, Scatolini, Pereira, Monteiro-Neto, cols; paratypes 1 whole juvenile CCUFLA 465 (same collection data as holotype).

Diagnosis: Body surface smooth. Epimeron 3 postero-distal margin acute. Eyes present, normal. Antenna 1 short, ~0.2× the body length. Antenna 2 half the body length. Maxilla 1 palp shorter than half the distance between the base of the palp and the base of setae on outer plate with setules and 1 small seta. Gnathopod 1 of male with polygonal pattern on the disto-posterior corner and comb scales on the anterior margin of the propodus, and polygonal pattern on the dactylus. Gnathopod 2 palm with and excavation, slope oblique, polygonal pattern on the disto-posterior corner. Pereopods 3–5 subequal in size. Uropod 3 peduncle and ramus subequal in length. Coxal gills on segments 2–6. Sternal gills on segments 2–7.

Male: Body surface smooth. Epimeron 3 postero-distal posterior margin acute. Coxae 1–3 subequal in size, same shape, slightly overlapping. Eyes present.

Antenna 1 (Fig. 7A) 0.23× the length of the body length and almost half (0.46×) the length of antenna 2; peduncle 1.2× longer than the head; peduncle segments 1–3 subequal in length; flagellum with 9 articles, 1.1× longer than peduncle; aesthetascs occurring distally on flagellum after article 4.

Antenna 2 (Fig. 7B) subequal to half the body length; peduncle 5-articulated, slender, 5.7× longer than head, article 5 longer and thinner than article 4; flagellum with 17 articles, the last one reduced, 1.8× longer than peduncle.

Mandibule without palp; incisor toothed; left (Fig. 7C) lacinia mobilis with 4 teeth, setae row with 2 long and 2 short strong pappose setae, molar process cylindrical, with 1 accessory seta; right mandible (Fig. 7D) with 1 long and 1 short pappose setae, molar process cylindrical, with 2 accessory setae.

Upper lip (Fig. 7E) margin round, distal margin covered with setules apically; lower lip (Fig. 7F) outer lobes rounded, slightly excavated, few setules disto-ventrally.

Maxilla 1 (Fig. 7H) inner plate slender bearing 2 apical pappose setae, setules laterally; outer plate with 9 serrate setae, few setules apically on the inner face; palp short, uniarticulate, longer than wide, not reaching half the distance between base of palp and base of setae on outer plate, with 1 apical seta and several setules distally.

Maxilla 2 (Fig. 7G) inner plate shorter than outer plate; inner plate with up to 12 papposerrate seta, the most basal one is the strongest, and up to 21 simple setae, apically, scattered setules on both margins; outer plate with many simple setae, various lengths, apically, and some setules on inner margin.

Maxilliped (Fig. 7I) without comb scales; inner plate with 3 cuspidate setae and some pappose seta apically; outer plate with 2 pappose setae apically and several simple seta distally and apically on inner margin; palp longer than inner plate, 4-articulated; article 1 inner margin shorter than outer margin, with 4 simple setae, outer margin with 1 simple seta; article 2 elongated, inner and apical margins with several scattered simple setae; article 3 elongated, apical-inner margin with some short and long simple setae, and up to 7 pappose setae, apical-outer margin with 3 simple setae and up to 9 longer simple setae; article 4 (dactylus) unguiform, with 4 apical simple seta, distal nail present.

Gnathopod 1 (Fig. 8A) subchelate; coxal plate 1.8× wider than long, with up to 15 simple setae on the margin; basis with 1–2 simple seta dorsally, up to 3 papposerrate seta apically; ischium with 2 papposerrate setae apically; merus with 4–5 papposerrate seta on distal margin; carpus longer than wide, almost diamond shaped, with up to 7 papposerrate seta on one margin, 5 pappose seta on inner margin and pappose seta forming a scoop-like on the produced lateral distal lobe; propodus 1.4× longer than wide, hammer-shaped, 14 simple setae on disto-anterior margin and comb scales, up to 12 pappose seta on ventral margin, and 2 pappose seta on disto-posterior margin, with polygonal pattern; palm slope transverse, with a few simple setae and 2 long pappose setae; postero-distal corner with 2 strong cuspidate setae; dactylus claw-like, with polygonal pattern on both margins, up to 3 simple seta on the tip and 1 plumose seta apically.

Gnathopod 2 (Fig. 8B) subchelate, without comb scales; coxal plate 1.8× wider than long, with 11 simple setae on the margin; basis with 1 or 2 papposerrate or simple setae laterally and/or distally; ischium and merus with papposerrate setae on posterior margin; carpus posterior lobe slim, produced between merus and propodus, forming a scoop-like structure, margin with pappose setae; propodus ovate, 1.4× longer than wide, postero-distal corner with polygonal pattern and 2 cuspidate setae with accessory seta, with a cup for the dactylus, palm slope oblique, with several cuspidate setae with accessory seta, longer than posterior margin; antero-distal margin with some long simple seta, 1 simple seta distally on anterior margin; dactylus claw like, congruent with the palm, 1 plumose seta distally.

Pereopods 3–7 (Fig. 8C–G) simple. Coxa 3 similar to 1 and 2, with up to 18 simple seta distally; coxa 4 1.2× longer than wide, excavated posteriorly, with up to 20 simple setae distally; coxa 5 posterior lobe as long and wide as anterior lobe, no observed seta; coxa 6 anterior lobe reduced, anterior lobe with 1 simple seta; coxa 7 reduced, up to 2 simple setae. Pereopods 3, 4 and 5 subequal in size, P4 slightly smaller; pereopods 3 and 4 setation similar, with 1 simple seta on the posterior margin of the basis, 5 groups of 1 to 3 simple setae on the posterior margin of the merus, and 5 or 6 groups of 1 to 6 cuspidate setae with accessory seta and simple setae on the posterior margins of carpus and propodus, dactylus with a plumose seta. Pereopods 5 to 7 setation similar, with several simple setae on the posterior margin of the basis and 4 to 6 groups of 1 to 3 cuspidate setae with accessory seta on the anterior margin of the basis, 2 to 3 groups of 2 to 5 cuspidate setae with accessory seta and simple seta on the anterior margins of the merus, carpus and propodus, 1 or 2 cuspidate setae with accessory seta on the posterior margin of the merus and a plumose seta on the dactylus. Pereopod 6 subequal in length to P7, ~1.3× longer than P3–P5.

Pleopods (Fig. 9A) peduncle shorter than both rami, with 2 coupling spines; both rami with several long plumose setae.

Uropod 1 (Fig. 9B) peduncle longer than both rami, with 2 simple setae on ventral margin and 2 cuspidate setae with accessory seta apically; inner ramus with 2 cuspidate setae with accessory seta distally and 3 short and 3 long cuspidate setae, 2 with accessory seta, apically, without a curved seta; outer ramus with 3 cuspidate setae with or without accessory setae distally and 4 cuspidate setae apically.

Uropod 2 (Fig. 9C) peduncle subequal to rami, with 1 simple seta on ventral margin and 1 cuspidate seta with accessory seta apically; inner ramus with 3 cuspidate setae with accessory seta distally and 5 cuspidate setae apically, one of them with accessory seta, 2 smaller and 2 longer; outer ramus with 3 cuspidate setae with or without accessory seta distally and 4 cuspidate setae, 2 smaller and 2 longer, 1 with accessory seta apically.

Uropod 3 (Fig. 9D) half the size of peduncle of uropod 1, subequal to the peduncle of uropod 2, with up to 7 long cuspidate setae with accessory seta; inner ramus absent; outer ramus uniarticulate, subequal to the peduncle, with 5 long simple setae and 1 short cuspidate seta.

Telson (Fig. 9E) entire, almost triangular, apically rounded with 2 symmetrical long simple setae apically, without lateral setae.

Epimeron (Fig. 7J) 1 and 2 postero-distal margin subacute; epimeron 3 postero-distal margin acute; the second is the greater in size and the third the smallest.

Coxal gills sac-like on pereonites 2–6; sternal gills tubular on pereonites 2–7.

Habitat: freshwater, hypogean, troglophile. The specimens of *Hyalella sp5* n. sp. were found buried on clay and sand substrate, on lotic and lentic pools inside “Gruta Jane Mansfield”.

Conservation: H. sp5 n. sp. is classified as endangered (EN) based on IUCN subcategory B1 (< 5.000km^2 in area of occurrence extension) and D1 (< 250 mature individuals) (ICMBIO, 2013).

Taxonomic remarks: When comparing *H. sp5* n. sp. with other cave species of *Hyalella*, the first difference we note is the presence of eyes. For those species with eyes, or with populations with variable eye presentation (*i. e.* specimens without eyes, with reduced eyes and with regular eyes in the same population), *H. sp5* n. sp. is different from *H. spelaea* on the relative size of antenna 2 in relation to the body length and the relative size of the palp of maxilla 1; it is different from *H. veredae* because the new species lacks a curved seta on the inner ramus of the male’s uropod 1; and it is different from *H. temimina* Penoni, Deotti & Bueno (Deotti *et al.*, in press) on the position of sternal gills and the relative length of the palp of maxilla 1. The pictorial key proposed on the next session of this paper provides an easier way to identify the subterranean *Hyalella* species from Brazil.

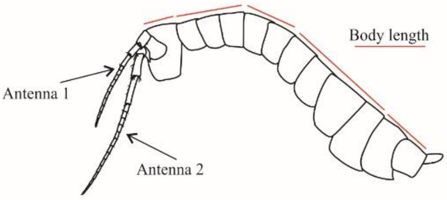
Pictorial key

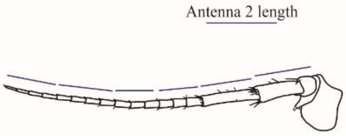
Hyalella Smith, 1874: Pictorial Key to the Brazilian Subterreneam Hyalellids

Ludmila R. Penoni & Alessandra A. P. Bueno

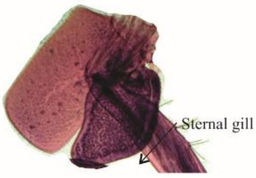
1. a) Eyes present ………………………………………………………………. 2
   b) Eyes absent, reduced or sometimes present ..……………………………….. 7

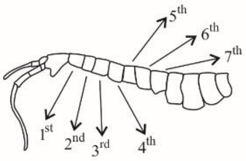
2. a) Antenna 2 longer than half the body length ………………….….…*Hyalella* sp2
   b) Antenna 2 not reaching half the body length ………………………………... 3



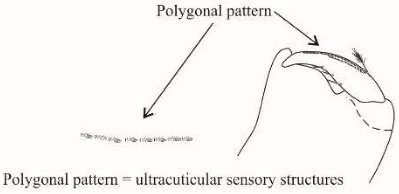


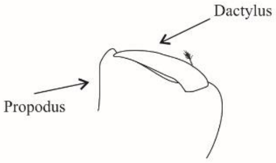
3. a) Coxal gills present on coxae 2 – 6 ……………………………………….....… 4
   b) Coxal gills present on coxae 2 – 7 …………………………….. *H. troglofugia*





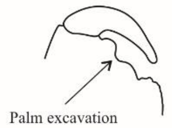
4. a) Gnathopod 1 dactylus with polygonal pattern ………...…………...… *H. caeca*
   b) Gnathopod 1 dactylus without polygonal pattern …………………………… 5

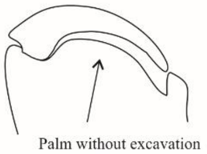




5. a) Gnathopod 2 palm with posterior excavation ………………………. *H. imbya*

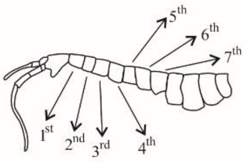
b) Gnathopod 2 palm without posterior excavation ……………………………. 6





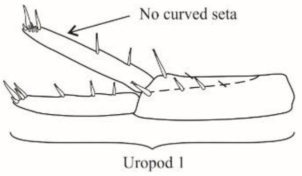
6. a) Sternal gills present on thoracic segments 2 – 7 ………………….. *H. formosa*

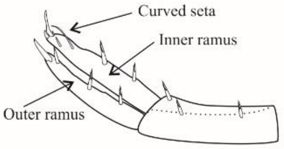
b) Sternal gills present on thoracic segments 3 – 7 ……………..… *H. epikarstica*



7. a) Male uropod 1 inner ramus with a curved seta ………………….... *H. veredae*

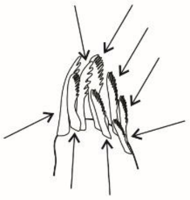
b) Male uropod 1 inner ramus without a curved seta ………………………….. 8

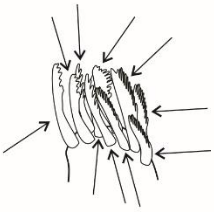




8. a) Maxilla 1 outer plate with 8 – 9 serrate setae 9

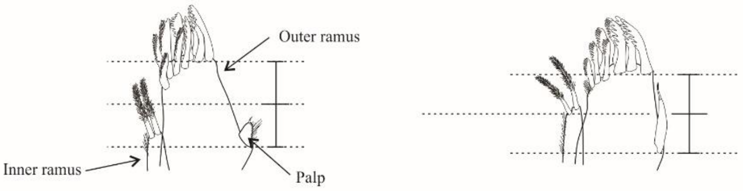
b) Maxilla 1 outer plate with 10 serrate setae ....*H.*sp4 n. sp.





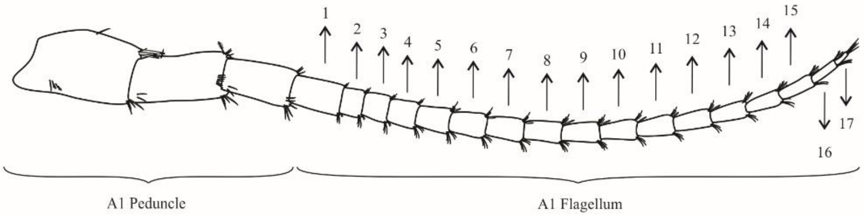
9. a) Maxilla 1 palp not reaching half the distance between the base of the palp and the base of the setae on the outer plate ....*H.* sp5 n. sp.

b) Maxilla 1 palp subequal or longer than half the distance between the base of the palp and the base of the setae on the outer plate 10



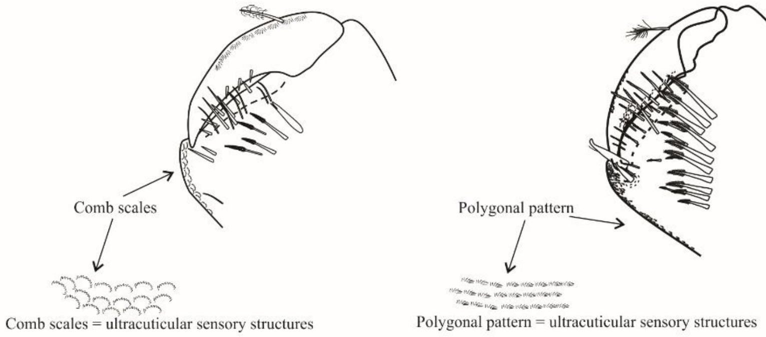
10. a) Antenna 1 flagellum with less than 10 articles ……………..……… *H. spelaea*

b) Antenna 1 flagellum with more than 10 articles ………....……………….... 11



11. a) Gnathopod 1 propodus with comb scales ……………………………… *H.* sp1

b) Gnathopod 1 propodus with polygonal pattern ……………….. *H. temimina*



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References

Araújo, P. B., Ferreira, R., Bueno, A. A. D. P., & Cardoso, G. M. (2014). Two new subterranean species of *Hyalella* Smith, 1874 (Crustacea: amphipoda: Hyalellidae) from Brazil. *Zootaxa, 3814(3)*, 353-368.

Bastos-Pereira, R., De Oliveira, M. P. A., & Ferreira, R. L. (2018). Anophtalmic and epigean? Description of an intriguing new species of *Hyalella* (Amphipoda, Hyalellidae) from Brazil. *Zootaxa, 4407(2)*, 254-266.

Bulycheva, A. (1957) The sea fleas of the USSR and adjacent waters (Amphipoda: Talitroidea). Keys to the Fauna of the USSR. *Akademiia Nauk SSSR, Opredeliteli po Faune SSSR, 65*, 1–185 (in Russian).

Cardoso, G. M., Bueno, A. A. D. P. & Ferreira, R. L. (2011). A new troglobiotic species of *Hyalella* (Crustacea, Amphipoda, Dogielinotidae) from Southeastern Brazil. *Nauplius*, 19, 17-26.

Deotti, C.M., Penoni, L.R., Bichuette, M.E., & Bueno, A.A.P. In press. Description of two new species of the freshwater amphipod *Hyalella* Smith, 1874 (Amphipod, Hyalellidae) for two state parks in São Paulo, Brazil. *Nauplius*, in press.

ICMBIO (Instituto Chico Mendes de Conservação da Biodiversidade). (2013). *Aplicação de critérios e categorias da UICN na avaliação da fauna brasileira.* Version 2.0. ICMBIO, Brasilia, Brazil.

Latreille, P. A. (1816) Amphipoda. In: *Nouveau Dictionaire d'histoire naturelle, appliquée aux Arts, à l'Agriculture, à l'Économie rurale et domestique, à la Médecine, etc. Par une société de Naturalistes et d'Agriculteurs.* 2nd edition. Volume 1. Deterville, Paris. Pp. 467–469.

Limberger, M., Santos, S. & Castiglioni, D. D. S. (2022). *Hyalella luciae* (Crustacea, Amphipoda, Hyalellidae) – a new species of freshwater amphipod from Southern Brazil. *Zootaxa,* 5174(5), 568-582.

Lowry, J. K., & Myers, A. A. (2013). A phylogeny and classification of the Senticaudata subord. nov. Crustacea: Amphipoda. *Zootaxa, 3610(1)*, 1-80.

Penoni, L.R., Lares, M.M.A., & Bueno, A.A.P. (2021). Description of a new species of the freshwater amphipod *Hyalella* Smith, 1874 (Amphipoda: Hyalellidae) from southeastern

Brazil, with remarks on their population biology and reproduction. *Journal of Crustacean Biology, 41(3)*, ruab050. https://doi.org/10.1093/jcbiol/ruab050

Pereira, V. F. G. C. (1989). Uma nova espécie de anfípode cavernícola do Brasil: *Hyalella caeca* n. sp. (Amphipoda, Hyalellidae). *Revista Brasileira de Zoologia*, 6, 49-55.

Rangel, C., Silva, A. L. L., Siegloch, A. E., Limberger, M. & Castiglioni, D. D. S. (2022). First island species of *Hyalella* (Amphipoda, Hyalellidae) from Florianpolis, state of Santa Catarina, Southern Brazil. *Zootaxa*, 5116(1), 40-60.

Rocha, B. N., & Galvani, E. (2013). Microclima de ambientes cavernícolas: estudo de caso da gruta Jane Mansfield, Parque Estadual de Intervales, SP. *Anais do 32º Congresso Brasileiro de Espeleologia*.

Rodrigues, S.G., Bueno, A.A.P. & Ferreira, R.L. (2012) The first hypothelminorheic Crustacea (Amphipoda, Dogielinotidae, *Hyalella*) from South America. *ZooKeys, 236*, 65–80. https://doi.org/10.3897/zookeys.236.3930.

Rodrigues, S.G., Bueno, A.A.P. & Ferreira, R.L. (2014) A new troglobiotic species of *Hyalella* (Crustacea, Amphipoda, hyalellidae) whith a taxonomic key for the brazilian species. *Zootaxa, 3815 (2)*, 200–214. https://doi.org/10.11646/zootaxa.3815.2.2

Rogers, D.C., Magalhães, C., Peralta, M., Ribeiro, F.B., Bond-Buckup, G., Price, W.W., Guerrero-Kommritz, J., Mantelatto, F.L., Bueno, A., Camacho, A.I., González, E.R., Jara, C.G., Pedraza, M., Pedraza- Lara, C., Latorre, E.R., & Santos, S. (2020). Phylum Arthropoda: Crustacea: Malacostraca. In: *Thorp and Covich's freshwater invertebrates* (J.H. Thorp & D.C. Rogers, eds), pp. 809–986. Academic Press, London.

São Paulo/SMA. Instituto Florestal (IF). (2010). *Plano de Manejo espeleológico Caverna do Diabo*.

Smith, S.I. 1874. The Crustacea of the freshwaters of the United States. A synopsis of the higher freshwater Crustacea of the Northern United States, Appendix F. Natural History. *United States Commission of Fish and Fisheries, 2*, 637–661.

Figures:

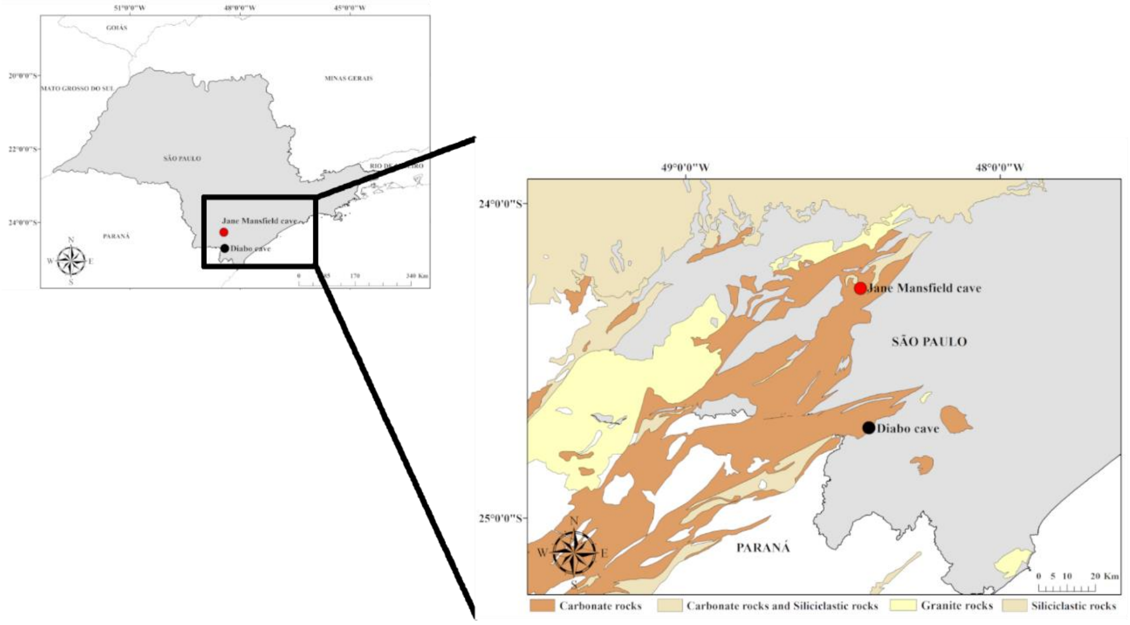

Figure 1: Map of collection sites of *Hyalella sp4* n. sp – Gruta da Tapagem Cave (“Caverna do Diabo”), Parque Estadual da Caverna do Diabo, Eldorado municipality, state of São Paulo, Brazil; and of *H. sp5* n. sp. – Jane Mansfield Cave, Parque Estadual Intervales, Iporanga municipality, state of São Paulo, Brazil.

Figure 2: Collection sites of *Hyalella sp4* n. sp. – “Caverna do Diabo”, Parque Estadual da Caverna do Diabo, Eldorado municipality, state of São Paulo. Hypogean collection site (**A**); epigean collection site (**B**).

Figure 3: *Hyalella sp4* n. sp., “Caverna do Diabo”, Parque Estadual da Caverna do Diabo, Eldorado municipality, state of São Paulo, Brazil. Holotype male, 6.84 mm (MNRJcarcino xxxxx) (**A**). Allotype female, 5.06 mm (MNRJcarcino xxxxx) (**B**). Scale bar = 1mm.

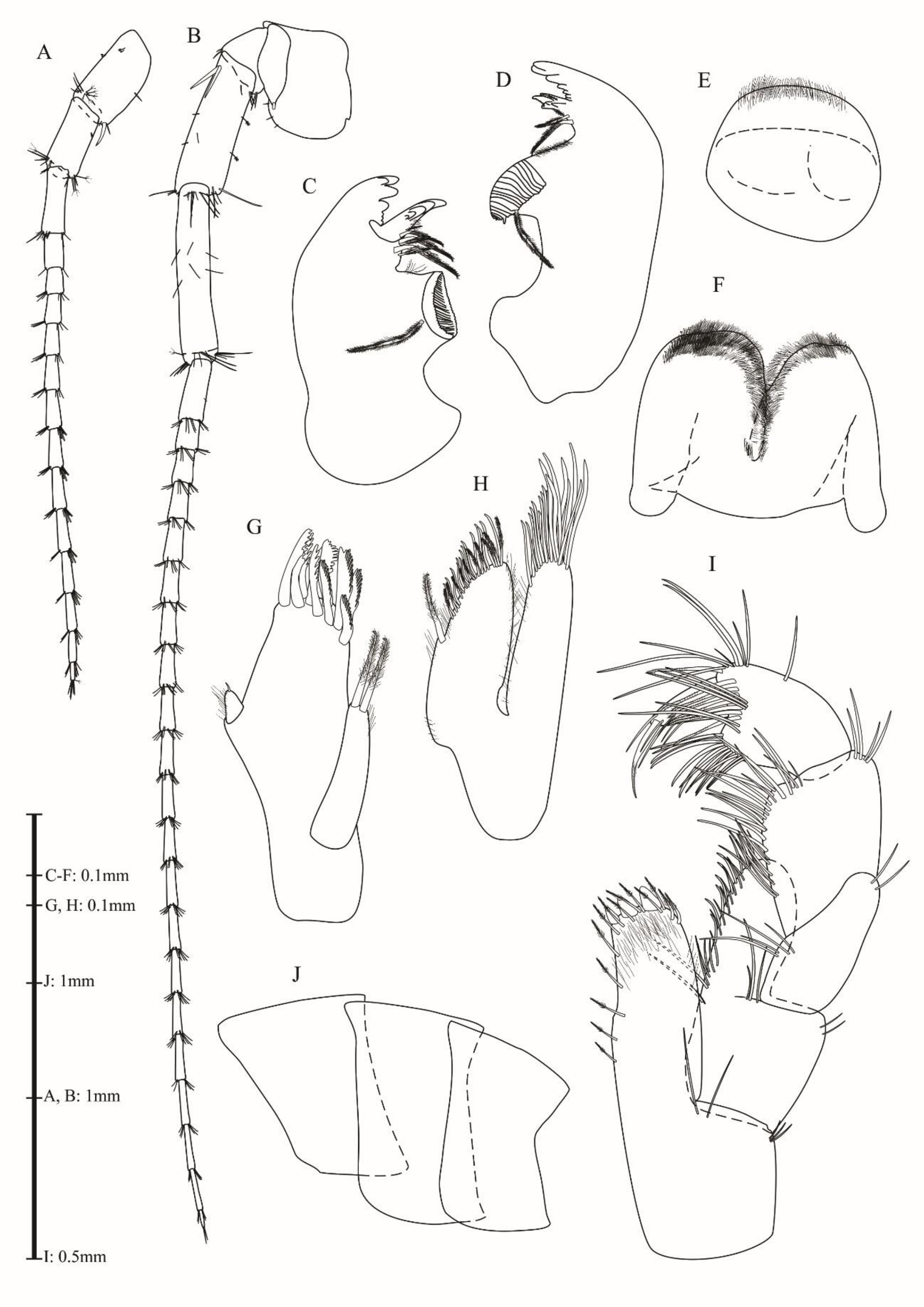

Figure 4: *Hyalella sp4* n. sp., “Caverna do Diabo”, Parque Estadual da Caverna do Diabo, Eldorado municipality, state of São Paulo, Brazil. Paratype male, 6.72 mm; antenna 1 (**A**); antenna 2 (**B**); left mandible (**C**); right mandible (**D**); upper lip (**E**); lower lip (**F**); maxilla 1 (**G**); maxilla 2 (**H**); maxilliped (**I**); epimeral plates (**J**). Scale bars: A, B and J= 1.0 mm; C–H = 0.1 mm; I = 0.5 mm.

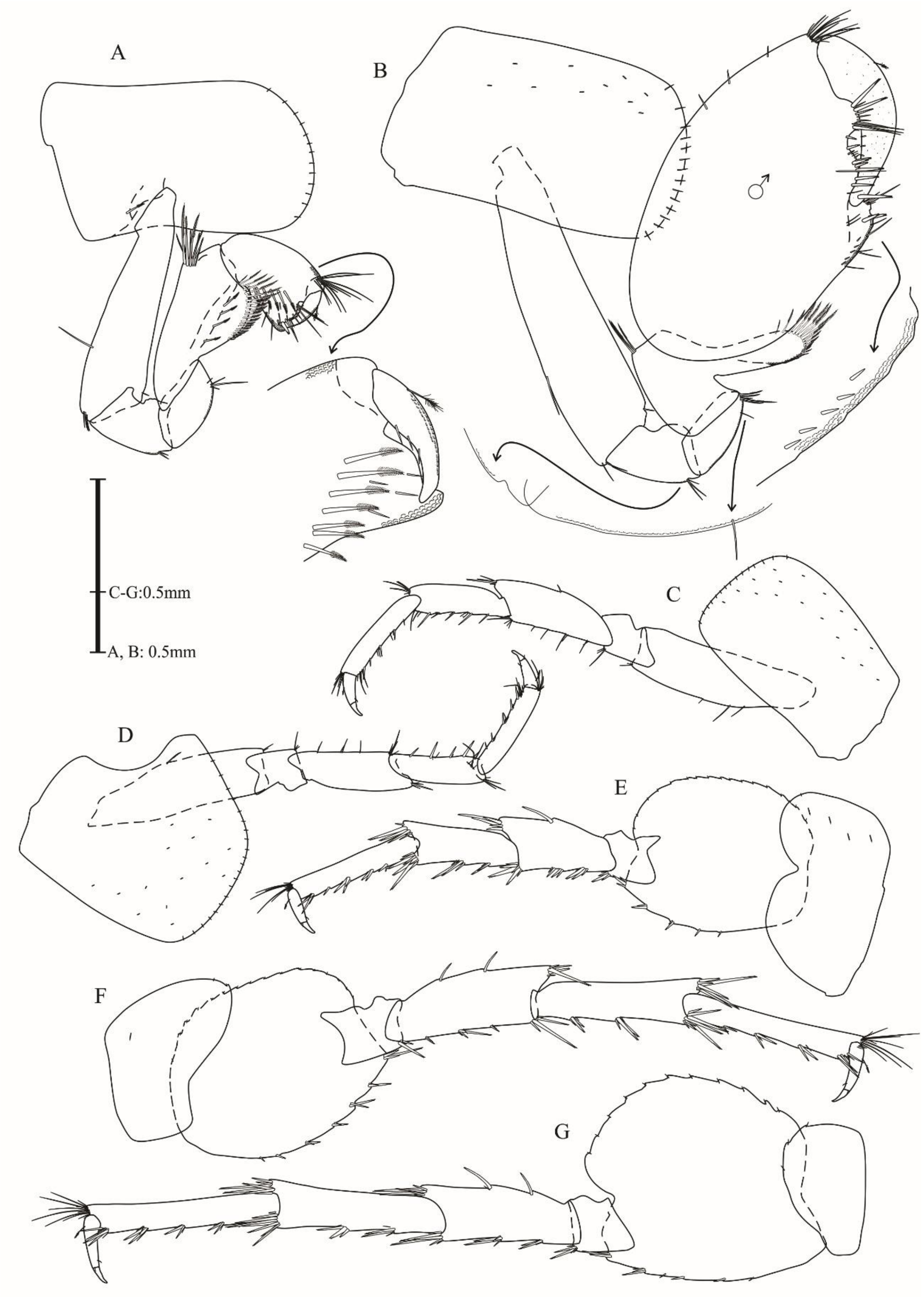

Figure 5. *Hyalella sp4* n. sp., "Caverna do Diabo", Parque Estadual da Caverna do Diabo, Eldorado municipality, state of São Paulo, Brazil. Paratype male, 6.72 mm; gnathopod 1 (**A**); gnathopod 2 (**B**); pereopod 3 (**C**); pereopod 4 (**D**); pereopod 5 (**E**); pereopod 6 (**F**); pereopod 7 (**G**). Scale bars = 0.5 mm.

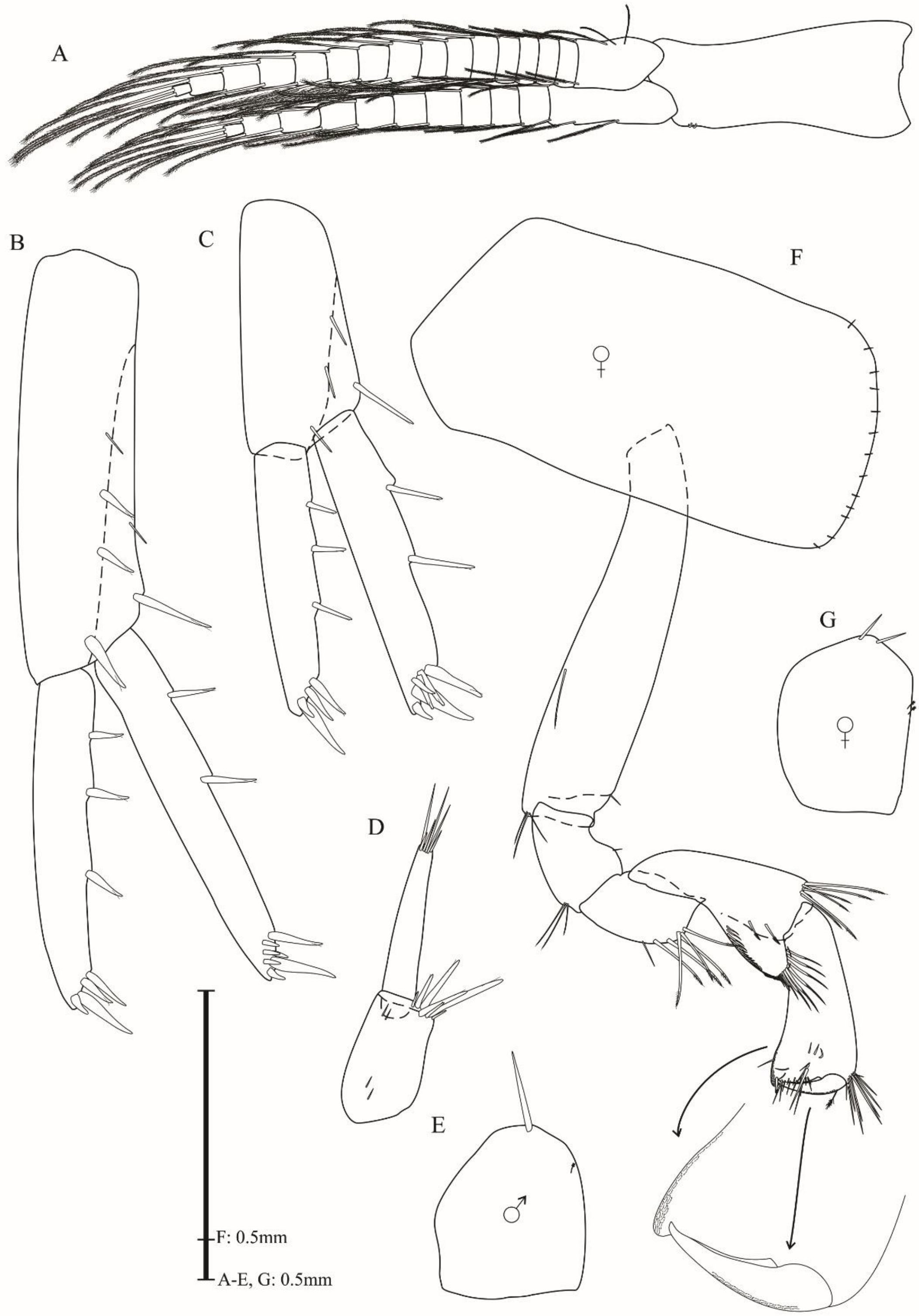

Figure 6. *Hyalella sp4* n. sp., “Caverna do Diabo”, Parque Estadual da Caverna do Diabo, Eldorado municipality, state of São Paulo, Brazil. Paratype male, 6.72 mm, paratype female, 6.18 mm; pleopod (**A**); uropod 1 (**B**); uropod 2 (**C**); uropod 3 (**D**); male telson (**E**); female gnathopod 2 (**F**); female telson (**G**). Scale bars = 0.5 mm.

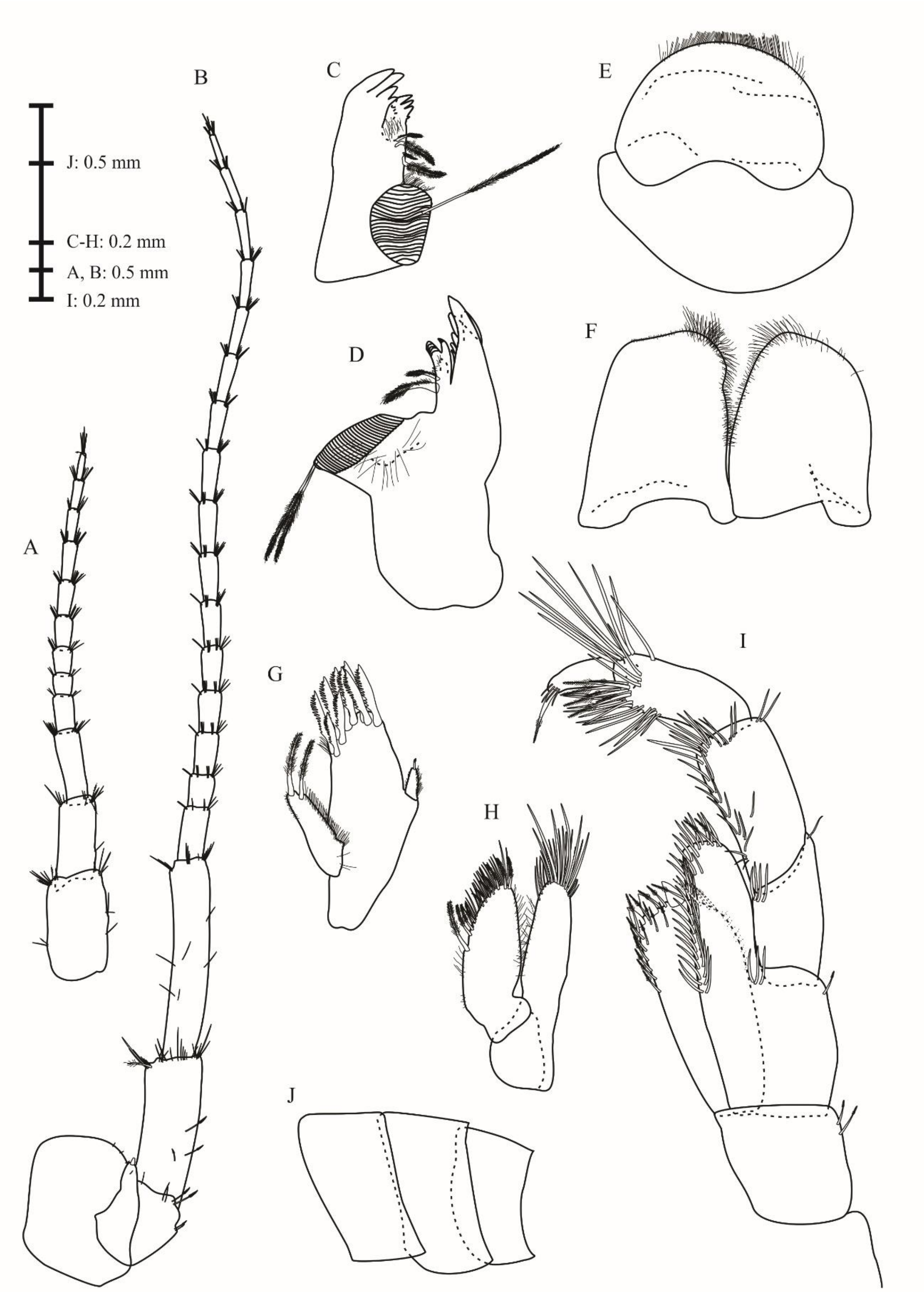

Figure 7: *Hyalella sp5* n. sp., Jane Mansfield Cave, Parque Estadual Intervales, Iporanga municipality, state of São Paulo, Brazil. Paratype male, 6.91 mm; antenna 1 (**A**); antenna 2 (**B**); left mandible (**C**); right mandible (**D**); upper lip (**E**); lower lip (**F**); maxilla 1 (**G**); maxilla 2 (**H**); maxilliped (**I**); epimeral plates (**J**). Scale bars: A, B, and J = 0.5 mm; C–I = 0.2 mm.

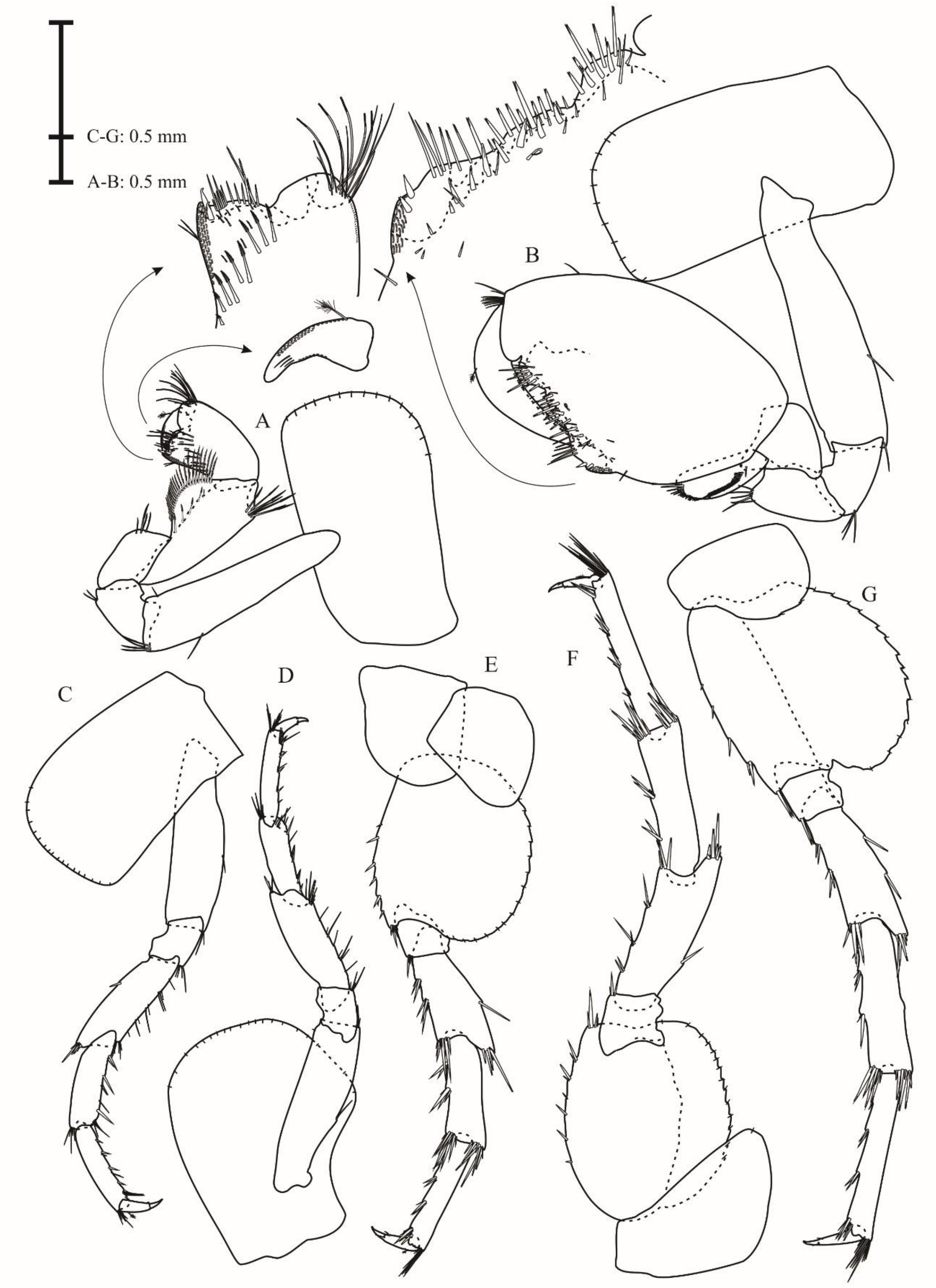

Figure 8. *Hyalella sp5* n. sp., Jane Mansfield Cave, Parque Estadual Intervales, Iporanga municipality, state of São Paulo, Brazil. Paratype male, 6.91 mm; gnathopod 1 (**A**); gnathopod 2 (**B**); pereopod 3 (**C**); pereopod 4 (**D**); pereopod 5 (**E**); pereopod 6 (**F**); pereopod 7 (**G**). Scale bars = 0.5 mm.

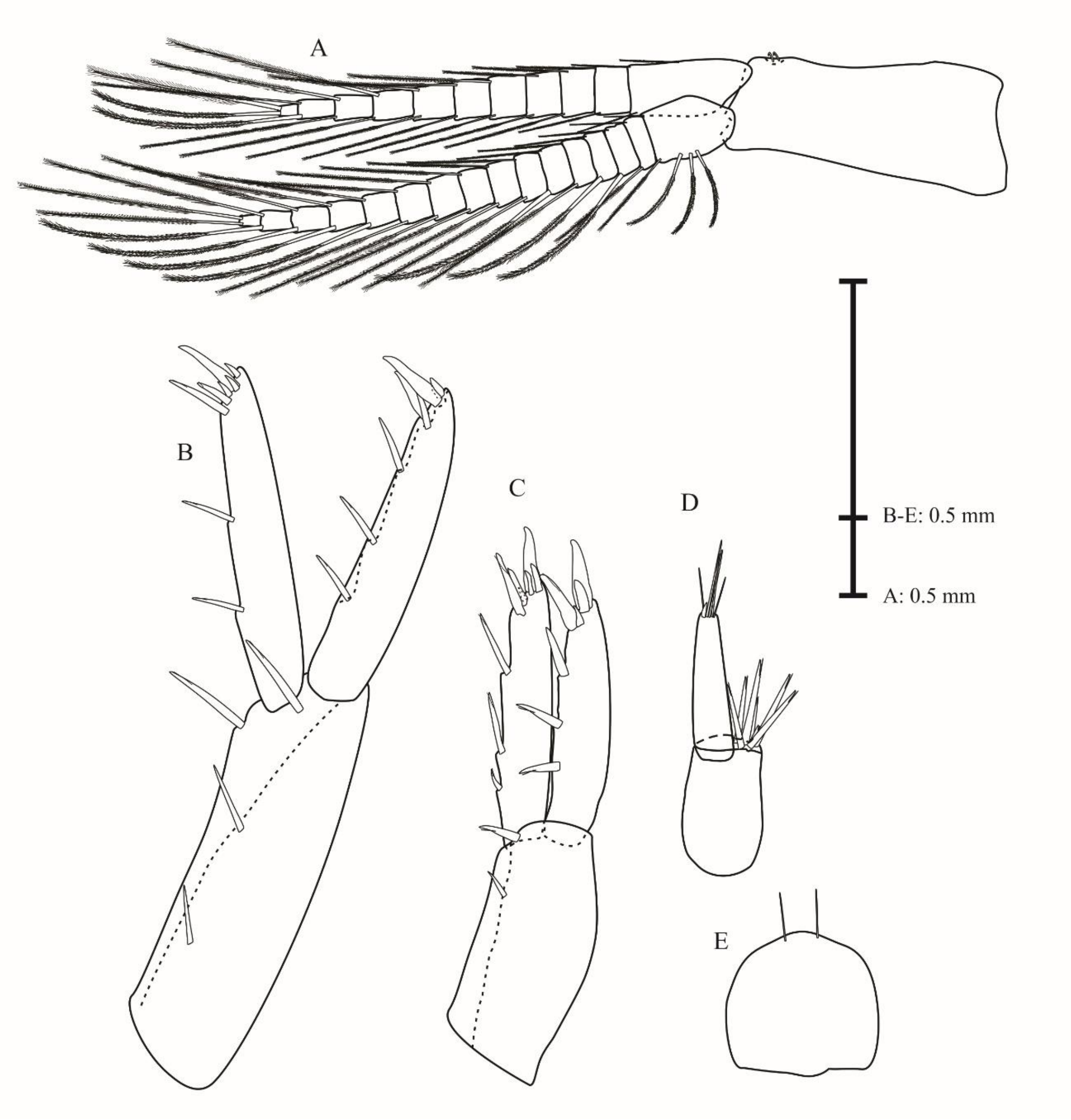

Figure 9. *Hyalella sp5* n. sp., Jane Mansfield Cave, Parque Estadual Intervales, Iporanga municipality, state of São Paulo, Brazil. Paratype male, 6.91 mm; pleopod (**A**); uropod 1 (**B**); uropod 2 (**C**); uropod 3 (**D**); telson (**E**). Scale bars = 0.5 mm.

5. Considerações finais

Esta tese é o resultado direto de 4 anos de pesquisa dedicados à ampliação dos conhecimentos sobre o gênero *Hyalella*, com especial foco na descrição de nova espécies do gênero associadas a cavernas da região sudeste do Brasil. É também resultado indireto de 12 anos – incluindo graduação, mestrado e doutorado – de estudos, aprendizados e desenvolvimento de técnicas e protocolos para uma eficiente descrição de *Hyalella*.

Como considerações finais, temos:

- *Hyalella* é um gênero especioso, abundantemente distribuído pelo Brasil; apesar do exponencial crescimento na diversidade do gênero nos últimos anos, ainda há muitas espécies a serem descobertas e descritas;

- Muitas espécies de *Hyalella* são endêmicas e restritas a pequenas áreas de ocorrência;

- A morfologia do gênero, embora extremamente conservada entre as espécies, varia sutilmente entre elas; maiores diferenças são observadas entre espécies simpátricas do que dentro do gênero de forma geral;

- O gênero *Hyalella*, pelo menos no sudeste brasileiro, teve diversas oportunidades para especiação ao longo do tempo evolutivo, provavelmente por alopatria e parapatria;

- A formação de especialistas em taxonomia e sistemática de *Hyalella*, com habilidades nas áreas de morfologia e análises moleculares é de grande importância para o reconhecimento da diversidade de espécies do gênero como um todo.