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RESEARCH ARTICLE

Hyperspectral analysis of color polymorphism and its variation in freshwater planarians (Platyhelminthes, Tricladida)

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Abstract

Many free-living turbellarians (Platyhelminthes, Turbellaria), including planarians (Tricladida), are polymorphic. However, the mechanisms underlying the intraspecific variation and spatial dynamics of their color polymorphism remain unclear and widely debated. In this study, the dorsal coloration of freshwater planarians from different ecological niches was analyzed using hyperspectral analysis. The planarians examined included five species endemic to Lake Baikal and three species typical of inland freshwaters in European Russia and Siberia. Hyperspectral measurements revealed significant interspecific differences. The results suggest that hyperspectral analysis can be used for the description of planarian phenotypic differences between biotopes as well as for the rapid identification of species.

Keywords

Bioindicator species, color polymorphism, endemic species, hyperspectral imaging, Lake Baikal, planarians

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Introduction

Free-living turbellarians (Platyhelminthes, Turbellaria) often exhibit significant variations in dorsal coloration, particularly pronounced in the orders Polycladida and Tricladida. Among them, marine polyclads, like many other reef-dwelling organisms, are the most brightly colored (Soutullo et al. 2021; Cuadrado et al. 2024; Pérez-García et al. 2024), whereas triclads, especially terrestrial geoplanids (Geoplanidae), are characterized by numerous color combinations and shades (Lago-Barcia et al. 2021; Justine et al. 2022). This diversity is also evident in freshwater triclads from the Caspian Sea (Dyganova 1982), Lake Ohrid (Stanković 1969), and Lake Baikal (Livanov 1961; Porfirieva 1977).

Taking into account the potential of planarians as bioindicators in ecotoxicological research due to their sensitivity to changes in environmental conditions and rapid response to the presence of pollutants in waters (Dyganova 1995; Tiras and Aslanidi 2013; Wu and Li 2018), an objective instrumental assessment of their phenotypic characteristics is required. Hyperspectral imaging represents a modern and powerful contactless tool for a quantitative assessment of coloration patterns, and thus could be instrumental for the rapid identification of triclad species and explicit characterization of their color polymorphism in both population and potentially biomonitoring studies.

Here, we suggest an instrumental approach for the intraspecies differentiation of planarians phenotypic features and species recognition based on hyperspectral imaging of their dorsal surface. The method was tested in freshwater triclads harvested from different ecological habitats.

In this work, the following planarians were examined: five species endemic to Lake Baikal (*Baikalobia guttata* (Gerstfeldt, 1858), *Baikalobia variegata* (Korotneff, 1912), *Baikalobia copulatrix* (Korotneff, 1912), *Bdellocephala angarensis* (Gerstfeldt, 1858), and *Hyperpapillina ocellata* (Korotneff, 1912)), and three species ubiquitous in various inland freshwater habitats in European Russia and Siberia (*Ijimia tenuis* Ijima, 1884, *Bdellocephala punctata* (Pallas, 1774), *Dendrocoelum lacteum* (Müller, 1774)). The triclads endemic to Lake Baikal are of particular interest because of their extensive dorsal color polymorphism that remains largely unexplained to date. The dorsal side of *B. guttata*, *B. variegata*, and *Bd. angarensis* is bright and mottled. In *B. copulatrix* and *H. ocellata*, it is dark umber and dark brown. On the contrary, *I. tenuis*, *Bd. punctata*, and *D. lacteum* exhibit relatively uniform dorsal coloration. The body surface of *D. lacteum* is pigment-deficient and exhibits only moderate color shading that varies depending on the diet (Dyganova and Porfirieva 1990). *B. guttata* exhibits pronounced color polymorphism and high ecological plasticity, inhabiting the entire shoreline of Lake Baikal with considerable variations in water temperatures (Porfirieva 1977).

Materials and methods

Planarians from different ecological habitats were used in the analysis: *I. tenuis*, *Bd. punctata*, and *D. lacteum* represent typical Palearctic species, while *B. guttata*, *B. variegata*, *B. copulatrix*, *Bd. angarensis*, and *H. ocellata* are endemic to Lake Baikal.

Baikal endemics were collected manually during the circum-Baikal expeditions (supervised by Prof. Oleg A. Timoshkin) onboard the R/V "Akademik V.A. Koptyug" in 2015, 2018, 2020. The collected samples were carefully harvested from the lower surface of stones located in shallow water along the shore with a soft brush to minimize damage and imaged alive using a standard RGB camera prior to being fixed in 70% ethanol. Collection dates and sampling sites with specific geographic coordinates are summarized in Table 1. Other species were collected in 2019 (Republic of Tatarstan, Kazanka River) and 2026 (Kazan, Golubye lakes system). Six biological specimens of each species were collected. The fixation in ethanol allowed the movement and changes in body shape to be prevented while imaging, as well as the properties at the sampling time without changes during storage and transportation. Furthermore, ethanol is more optically transparent in the near-infrared band, and thus standardizes the analysis conditions.

Table 1. Geographic coordinates and descriptions of the planarian sampling sites

Species	Sampling locality and date	Geographic coordinates
Ijimia tenuis	February 26, 2026; Kazan, Golubye lakes system; depth 0.5 m	55°54.1080′N, 49°9.4018′E
Bd. punctata	August 10, 2019; Republic of the Tatarstan, oxbow of Kazanka River; depth 0.1 m	55°59.7271′N, 49°25.3195′E
D. lacteum	February 26, 2026; Kazan, Golubye lakes system; depth 0.5 m	55°54.1080′N, 49°9.4018′E
B. guttata	June 9, 2018; Lake Baikal, Bol'shoy Ushkaniy Island, Severnaya Bay; shoreline	53°51.8388′N, 108°37.3526′E
B. variegata	15 June 2020; Lake Baikal, Cape Berezovyi; depth 3 m	51°50.9635′N, 104°55.2685′E
B. copulatrix	10 June 2015; Lake Baikal, Cape Berezovyi; depth 1 m	51°50.9635′N, 104°55.2685′E
Bd. angarensis	10 June 2015; Lake Baikal, Cape Berezovyi; depth 1 m	51°50.9635′N, 104°55.2685′E
H. ocellata	June 17, 2020; Lake Baikal, Bol'shoy Ushkaniy Island, meteorological station; depth 6–10 m	53°51.0646′N, 108°36.0898′E

Hyperspectral images of ethanol-fixed planarians were obtained at the Hyperspectral Lab at St. Petersburg Electrotechnical University using a Specim FX10 camera (Specim Ltd., Oulu, Finland) in line-scanning mode, complemented by a motorized linear stage, a height-adjustable camera mount, and six halogen lamps arranged in two opposing rows. This configuration enabled uniform illumination across the imaging area and sequential image acquisition as the samples moved along the camera's scanning line. The design and characteristics of the hyperspectral acquisition system are further detailed in (Baranov 2024).

All specimens were placed in individual wells on a plate made of light-absorbing black plastic to minimize stray reflectance while providing sufficient contrast with the background. The wells were filled with an ethanol solution to keep the planarians partially submerged with their dorsal sides exposed for imaging.

Hyperspectral images were acquired at 400–1000 nm, yielding 224 layers each corresponding to a narrow spectral band characterized by 5.5 nm full width at half maximum (FWHM). The camera was placed at a height of 25 cm above the plate. The scanning stage moved at 0.8 mm/s. To increase the dynamic range, two images with different exposure times of 200 and 333 ms were acquired, aligned using a previously implemented image registration procedure (Vishnevskiy et al. 2017), and a high dynamic range (HDR) image was created by combining only linear parts of the sensor photon transfer characteristic below 75% of its saturation level following (Debevec and Malik 1997). The spatial resolution was about 100 μm per pixel. Camera operation, linear scanning, and hyperspectral data acquisition were controlled with Lumo Scanner 2020 software. Two independent HDR images (technical replicates) were acquired for each biological specimen, and averaged before further statistical analysis, while different biological specimens were treated as independent observations (n = 6 per species).

For further analysis, the reflectance measurements were renormalized using images of a dark and white reference target, the latter with a nominal reflectance of 80–95%. The acquired hyperspectral images were then manually segmented using ImageJ/Fiji software to locate individual planarian specimens, and their reflectance spectra were obtained by averaging over the selected image areas. To account for the intra-species coloring variability, spectral signatures were obtained and considered separately for each of the planarian specimens. Spectral data below 450 nm and above 950 nm were excluded from subsequent analysis because of elevated noise levels associated with low camera sensitivity in the respective spectral bands. To compensate for discrepancies in overall spectral intensity, the reflectance spectra were next normalized to their average intensities across the entire 450–950 nm spectral range. Statistical significance was assessed using the nonparametric Kruskal-Wallis test, followed by Dunn's post-hoc multiple comparison test. Differences were considered statistically significant at $p<0.05$.

Results

Visual examination of the triclad species from their prefixation images (Fig. 1) indicates considerable differences in their external appearance. More specifically, the dorsal side of *I. tenuis* is typically from dark umber to nearly black (Fig. 1A), with numerous eyes (up to 70) arranged in a single row along the nonpigmented margin of the anterior end (Dyganova and Porfirieva 1990). *Bd. punctata* exhibits a light brown dorsal background (Fig. 1B) with small spots scattered throughout the body and two eyes at the anterior end (Dyganova and Porfirieva 1990). The body of *D. lacteum* is pale (Fig. 1C) with almost transparent surface tissues, through which the intestine is visible, and two eyes at the anterior end.

B. guttata is characterized by mottled pigmentation with a complex dorsal pattern (Fig. 1D). Light-colored spots are asymmetrically distributed on the left and right sides of the medial axis, which is sometimes lighter than the surrounding body surface. Each lateral band contains about 7–9 spots. The eyes, from 7 to 9, reside on the inner side of each light-colored eye spot at the anterior end of the head (Porfiriev et al. 2018).

B. variegata (Fig. 1E) exhibits a bright and strongly mottled coloration on the dorsal side. The dorsal background is brownish orange with numerous small, irregular dark, and light spots. Two rows of rounded, light yellow to ochre spots flank the medial axis, with 6 to 7 spots per row, forming a typical dorsal pattern. Light-yellow strokes or bands run laterally from these spots to the lateral margins of the body. The eyes (12–15) are arranged in two archlike rows along the inner margins of the white eye spots (Porfirieva 1977).



Figure 1. External appearance of the studied freshwater planarians studied photographed in vivo. (**A–C**) Palearctic species: *Ijimia tenuis* (**A**), *Bdellocephala punctata* (**B**), and *Dendrocoelum lacteum* (**C**). (**D–H**) Lake Baikal endemic species: *Baikalobia guttata* (**D**), *B. variegata* (**E**), *B. copulatrix* (**F**), *Bdellocephala angarensis* (**G**), and *Hyperpapillina ocellata* (**H**). Specimens were imaged before ethanol fixation to preserve and document their natural coloration and morphological features. Scale bars = 5 mm.

B. copulatrix (Fig. 1F) has a dark gray to nearly black dorsal side, with intestinal branches sometimes visible through the dark background. Eyes (5-9) occur along the inner margins of two light-colored ovals at the anterior end of the body. *Bd. angarensis* is ochreous brown on the dorsal side, with numerous irregular dark brown spots or strokes (Fig. 1G). The head bears two large eyes near the inner margins of the light-colored spots. The dorsal side of *H. ocellata* is uniformly dark brown to nearly black with a reddish hue and a white transverse collar at the anterior end (Fig. 1H). Two clusters of eyes, each consisting of more than 15 eyes, are located on the collar (Porfirieva 1977).

Figure 2 shows the individual reflectance spectra of four planarian species: *B. guttata*, *Bd. punctata*, *I. tenuis*, and *D. lacteum* (Figs. 1A-D). Of these, *B. guttata* is endemic to Lake Baikal, whereas the other three species are ubiquitous in the freshwater bodies of European Russia.

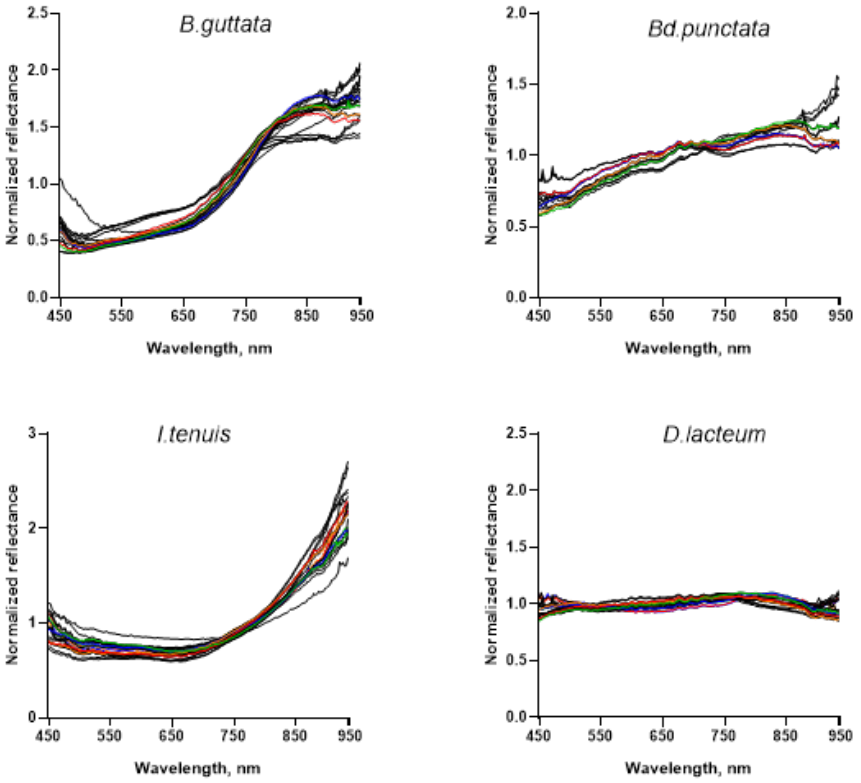


Figure 2. Individual reflectance spectra of the dorsal surfaces for four planarian species. Each line represents the normalized reflectance spectrum of an individual biological specimen (n = 6 per species). The panels display the spectral profiles for the Lake Baikal endemic *Baikalobia guttata* and three Palearctic species: *Bdellocephala punctata*, *Ijimia tenuis*, and *Dendrocoelum lacteum*. Spectral data were acquired in the 450–950 nm range.

The figure shows that the observed intraspecies reproducibility indicates relatively low variability of the normalized spectral profiles among specimens of the same species and may partially reflect similarities in dorsal pigmentation among specimens of the same species. Among them, *D. lacteum* was the only species that exhibited a nearly flat reflectance spectrum across both visible and near-infrared (NIR) ranges, indicated by normalized reflectance values close to one, characterizing the absence of detectable pigments that led to their optical whiteness. On the contrary, *B. guttata*, *I. tenuis*, and *Bd. punctata* exhibit complex spectral profiles that represent characteristic pigmentation patterns of their dorsal tissues in the spectral domain.

Despite moderate individual variability in their spectral signatures, the overall shapes of the reflectance spectra remained reproducible for a given species, indicating hyperspectral imaging and analysis as a potential tool for species-level identification of triclads.

Figure 3 shows the median reflectance spectra of the same four planarian species (Fig. 1A–D), as well as their intraspecies variabilities. Significant discrepancies between normalized reflectance spectra of *I. tenuis* and *D. lacteum* could be observed within the 475–780 nm and 820–950 nm bands ($p_{adj}<0.00001$). Similarly, the spectral shapes of *I. tenuis* and *Bd. punctata* clearly differed within the 556–800 nm and 830–950 nm bands ($p_{adj}<0.00001$), suggesting that their pairwise identification could easily be reduced to the analysis of dual-band spectral indices. The entire visible spectral range, except for the 625–691 nm band ($p_{adj}<0.00001$), can be used to distinguish the normalized spectra of *I. tenuis* and *B. guttata*. Spectra from *B. guttata* and *D. lacteum* did not overlap, except at 718–745 nm ($p_{adj}<0.00001$), corresponding to the red region of the visible spectrum. To reliably separate *B. guttata* from *Bd. punctata*, the same spectral regions should be excluded. For *Bd. punctata* and *D. lacteum*, the interquartile ranges overlapped considerably, with the 450–583 nm and >773 nm spectral regions being the most informative to distinguish between them ($p_{adj}<0.00001$). In the NIR range, a significant overlap between three species could be observed around 780–820 nm ($p_{adj}<0.00001$), providing a potential reference for multispecies identification indices.

D. lacteum exhibited an approximately f lat normalized spectral response across the visible range because of nearly uniform reflection of the visible light by evenly light-colored surfaces. On the contrary, in the darker species, the spectral response often appeared more characteristic in the NIR range, because dark pigments absorbed visible light and reflect strongly in the IR region.

Together, the results presented above indicate that the reflectance spectra patterns are largely unique in each species, suggesting their reliable identification through hyperspectral analysis.

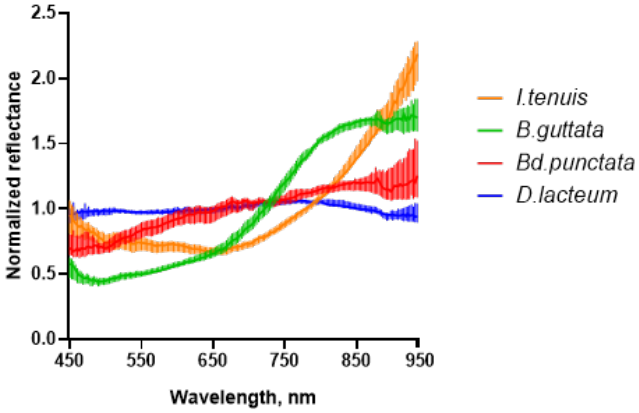


Figure 3. Median reflectance spectra with interquartile ranges (IQR) for four planarian species. Solid lines represent the median reflectance (n = 6 per species), and shaded regions indicate the interquartile range (IQR). The species include the Lake Baikal endemic *Baikalobia guttata* and three Palearctic species: *Bdellocephala punctata*, *Ijimia tenuis*, and *Dendrocoelum lacteum*.

Figure 4 shows the individual reflectance spectra of the four largest planarian species in our sample, all of different genera: *H. ocellata*, *Bd. angarensis*, *B. copulatrix*, and *B. variegata* (Fig. 1E–H). On visual inspection, two of them (*H. ocellata* and *B. copulatrix*) appear uniformly dark brown to nearly black, while the remaining two (*Bd. angarensis* and *B. variegata*) exhibit the most complex, mottled coloration among the studied species.

The spectral signatures in Figure 4 were used to assess intraspecies variability. Although the spectral curves of *H. ocellata* and *Bd. angarensis* are well reproduced between specimens, more pronounced intraspecies variability of the spectral profiles could be observed in *B. copulatrix* and *B. variegata*, reflecting the overall complexity of their dorsal coloration. In particular, pronounced inhomogeneity could be observed in the reflectance profiles across the considered spectral range, indicated by enhanced reflectance in the NIR band, typically 3 to 5-fold that of the reflectance in the blue and green bands. Again, nearly universal reflectance patterns across considered species could be observed above 800 nm, and especially around 900 nm.

Again, the above results indicate that planarian species exhibit a complex multitude of reflectance spectra ranging from nearly flat to highly inhomogeneous characteristics across visible and NIR bands.

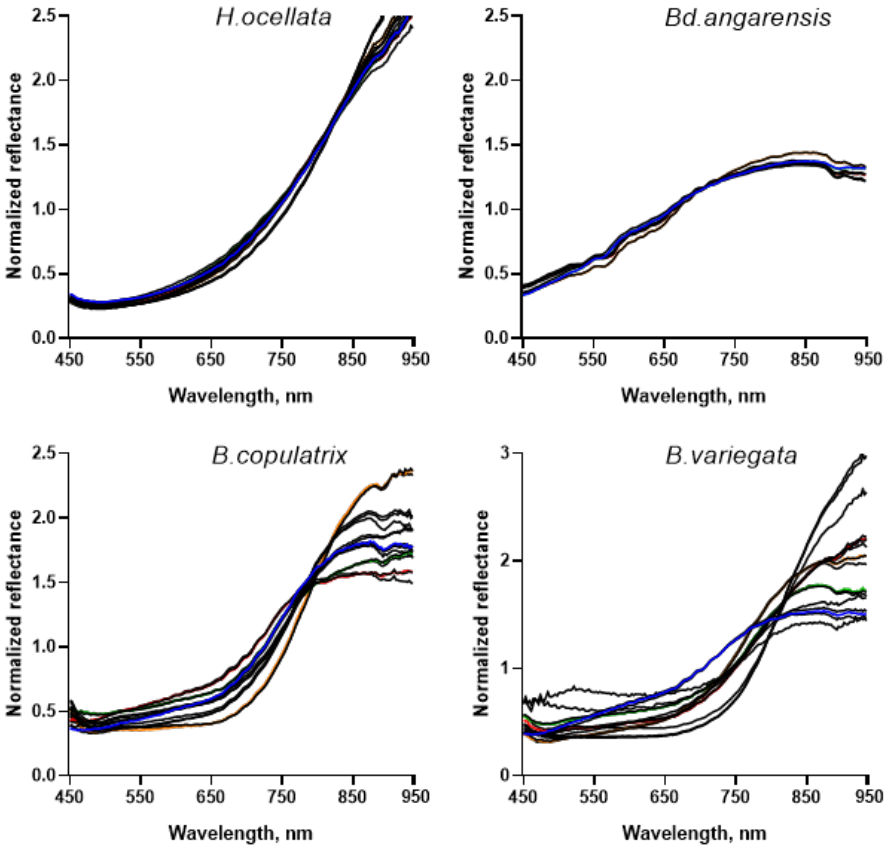


Figure 4. Individual reflectance spectra of the dorsal surfaces for four Lake Baikal endemic planarian species. Each line represents the normalized reflectance spectrum of an individual biological specimen (n = 6 per species). The panels show the spectral profiles for *Hyperpapillina ocellata*, *Bdellocephala angarensis*, *Baikalobia copulatrix*, and *Baikalobia variegata*.

Figure 5 shows the median spectra of the eight planarian species from different ecological habitats (Lake Baikal, the Golubye lakes system, and the Kazanka River). The figure shows that the reflectance spectra exhibit various patterns, from nearly flat for *D. lacteum* to moderately enhanced in the red-edge (RE) and NIR bands for *Bd. punctata* and *Bd. angarensis* to strongly suppressed in the visible, while pronounced only in the RE and enhanced in the NIR bands for *B. guttata*, *B. copulatrix*, *B. variegata*, and *H. ocellata*.

Despite the substantial overlap between the observed spectral profiles, there is a potential reference band with nearly universal normalized reflectance patterns around the 700–750 nm RE band, complemented by significant discrepancies be-

tween spectral profiles in the visible or in the NIR band, or both, for every pairwise combination of species. This fact allows for suggesting their possible identification via a pair of dual-band indices or a single three-band index, following a common approach in the multispectral image analysis widely adopted across multiple fields of science from general biology to ecology, agriculture, conservation science and beyond. However, these indices require evidence-based development and validation.

Given the high spectral resolution provided by hyperspectral imaging that considerably exceeds conventional multispectral technologies, the above approach could be potentially generalized toward a multiband classifier capable of identifying a large selection of species. Further information for identification and possibly also physiological status characterization could be obtained by complementing spectral and textural information embedded in the hyperspectral images.

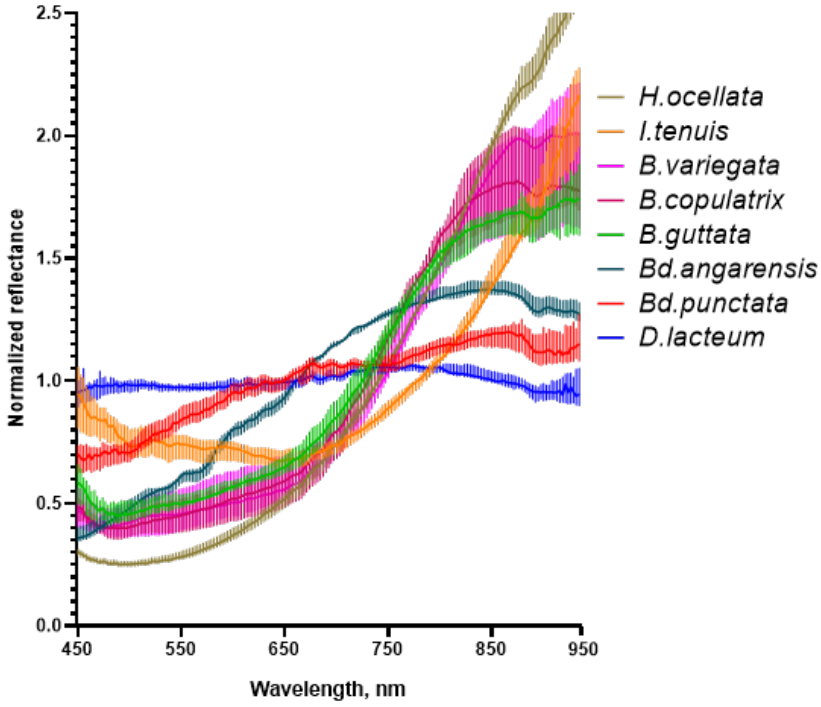


Figure 5. Comparative median reflectance spectra with interquartile ranges (IQR) for all eight studied planarian species. Solid lines represent the median reflectance (n = 6 per species), and shaded regions indicate the IQR. Species are grouped by ecological origin: Palearctic species (*Ijimia tenuis*, *Bdellocephala punctata*, *Dendrocoelum lacteum*) and Lake Baikal endemics (*Baikalobia guttata*, *B. variegata*, *B. copulatrix*, *Bdellocephala angarensis* and *Hyperpapillina ocellata*).

Among prominent examples, several variants of multivariate classifier designs from Bayesian networks (BN) to convolutional neural networks (CNN) based solely on spectral or both spectral and textural features proposed recently in the context of microbial species identification in (Bogachev et al. 2026) could be considered as potential prototypes of multivariate model design. Since training of the respective classification models would require a large collection of hyperspectral images of specimens obtained and acquired under similar controlled conditions to be available, we currently consider this as a promising avenue for future studies.

Discussion

To summarize, individual reflectance spectra of eight freshwater planarians from different ecological habitats were obtained from their hyperspectral images. Their comparative analysis revealed intraspecific and interspecific variability in their optical characteristics, which could be presumably attributed to the discrepancies in their dorsal pigmentation from complex, bright and mottled patterns (*B. guttata* and *B. variegata*) to nearly uniform milky white or nearly black coloration (*D. lacteum* and *I. tenuis*).

The degree of pigmentation was found to influence the optical structure. The most complex optical structures were identified in *B. guttata* (mottled), *I. tenuis* (uniform black), and *Bd. punctata* (light brown body surface with dark spots). The highest reflectance in the visible range was observed in *D. lacteum* (milky white) because light-colored surfaces reflect visible light evenly.

H. ocellata, *Bd. angarensis*, *B. copulatrix*, and *B. variegata* exhibited relatively uniform spectral patterns. *H. ocellata*, known for its morphological stability consistent with its visual appearance in a uniform dark-brown coloration, demonstrated the most pronounced spectral inhomogeneity, indicating that spectral characteristics are more informative in their distinction from other species. Somewhat similar features could be observed for *Bd. angarensis*: although mottled when alive, its pigmentation is very unstable and fades rapidly after fixation, possibly influencing the color variation visible in preserved specimens.

B. copulatrix exhibited a moderate divergence of its spectral signatures in the IR range, indicating variability in its optical properties. However, it is difficult to explain this in terms of external appearance (dark-umber coloration), and the mechanisms underlying the optical properties of the body surface in planarians require further investigation.

Unlike *Bd. angarensis*, the other triclad species used in the experiments retained their natural coloration after fixation. Therefore, the use of ethanol-fixed samples looks as a limit to the study because of possible changes in the reflectance properties of animals, apparent removal of any colored metabolites, and observed decolorization of some species. On the other hand, viable worms transform their body size

during movement, thus making simple hypercube assembly difficult without complicated processing and changing optical features of the body.

The origin of the pronounced color polymorphism observed on the dorsal side of Baikal planarians remains a complex and debated issue. Several hypotheses suggest that this complex coloration allowed the group's ancestral species to colonize the lake's littoral zone. It is also worth noting that the intricate patterning of Baikal planarians, particularly those of the genus *Baikalobia*, probably plays a protective role, helping flatworms blend in with the surrounding stones.

However, these data are preliminary and require further validation, as well as optimization of hyperspectral imaging.

While certain overlap between spectral patterns could be observed, significant intra-species variations either in the visible or in the NIR band or both for every pairwise combination of species indicate that they could be potentially identified from their spectral patterns only. In a more general scenario, the identification problem among a larger collection of species could be potentially generalized to multivariate classifiers, which we consider as a possible outlook.

Conclusion

Hyperspectral imaging was used to obtain the reflectance spectra of eight freshwater planarians from different habitats, revealing both intraspecific and interspecific variability of their optical characteristics. The degree of pigmentation influenced the optical structure, with the most complex structures found in mottled or uniformly dark species such as *B. guttata*, *I. tenuis*, and *Bd. punctata*. Light–colored species such as *D. lacteum* showed the highest reflectance in the visible range, while some species exhibited a notable spectral inhomogeneity that was not always aligned with their visible coloration. Significant intraspecies variations in the visible and NIR bands suggest that spectral patterns could potentially be used to distinguish between species. Multivariate classifiers can offer a viable approach for generalizing species identification in larger collections, pointing to a promising direction for future research.

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