

## SIMILARITIES AND DIFFERENCES BETWEEN THE *CHAETOPTERUS VARIOPEDATUS* POLYCHAETE LUCIFERASES DEPENDING ON THE TYPE OF HABITAT

Purtov KV<sup>1</sup>, Petushkov VN<sup>1</sup>, Rodionova NS<sup>1</sup>, Chepurnykh TV<sup>2</sup>, Kozhemyako VB<sup>3</sup>, Zagitova RI<sup>2</sup>, Shcheglov AS<sup>2,4</sup> ✉, Ziganshin RH<sup>2</sup>, Tsarkova AS<sup>2,4</sup>

<sup>1</sup> Institute of Biophysics, Krasnoyarsk Science Center of the Siberian Branch of the Russian Academy of Sciences, Russia

<sup>2</sup> Shemyakin–Ovchinnikov Institute of Bioorganic Chemistry, Moscow, Russia

<sup>3</sup> Pacific State Medical University, Vladivostok, Russia

<sup>4</sup> Pirogov Russian National Research Medical University, Moscow, Russia

The marine polychaete *Chaetopterus varioopedatus* (Renier) (family *Chaetopteridae*) is a cosmopolitan species complex, consisting of distinct populations/subspecies. The worms release glowing (460 nm) clouds of mucus when disturbed, and their parapodia often glow brightly. Currently, it is still unclear how exactly the bioluminescence system of these polychaetes functions. It has been previously assumed that the *C. varioopedatus* luciferase may be used for detection of ferroptosis, the recently explored pathway of programmed cell death, resulting from accumulation of the ferrous ions. This study was aimed to extract and characterize the *C. varioopedatus* luciferases, as well as to compare luciferases obtained from *C. varioopedatus* of different populations. When extracting the enzyme responsible for bioluminescence from the frozen samples of Brazilian *C. varioopedatus* using the improved method, two active luciferases, L1 and L2, were obtained. We assumed that one of the listed above luciferases was responsible for luminescence of the mucus and the other luciferase was responsible for luminescence in parapodia, and used the method for the distinct samples of mucus and parapodia of the living Far Eastern *C. varioopedatus*. However, mucus of the latter turned out to be non-glowing. It is shown that luciferase L2 is responsible for luminescence in the parapodia of the *C. varioopedatus* polychaete, since this luciferase has been found in the total biomass of Brazilian polychaetes and parapodia of Far Eastern polychaetes. Luminescence of the Brazilian *C. varioopedatus* mucus is attributed to the functioning of luciferase L1, which is lacking in the mucus of the Far Eastern subspecies. The range of luciferase isoforms in polychaetes *C. varioopedatus* depends on the place of origin.

**Keywords:** bioluminescence, luciferase, polychaetes, *Chaetopterus varioopedatus*, marine worms

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✉ **Correspondence should be addressed:** Aleksandr S. Shcheglov  
Miklukho-Maklaya, 16/10, Moscow, 117997, Russia; jukart@mail.ru

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## СХОДСТВО И РАЗЛИЧИЯ ЛЮЦИФЕРАЗ ПОЛИХЕТ *CHAETOPTERUS VARIOPEDATUS* В ЗАВИСИМОСТИ ОТ МЕСТА ИХ ОБИТАНИЯ

К. В. Пуртов<sup>1</sup>, В. Н. Петушков<sup>1</sup>, Н. С. Родионова<sup>1</sup>, Т. В. Чепурных<sup>2</sup>, В. Б. Кожемяко<sup>3</sup>, Р. И. Загитова<sup>2</sup>, А. С. Щеглов<sup>2,4</sup> ✉, Р. Х. Зиганшин<sup>2</sup>, А. С. Царькова<sup>2,4</sup>

<sup>1</sup> Институт биофизики, Красноярский научный центр Сибирского отделения Российской Академии наук, Россия

<sup>2</sup> Институт биорганической химии имени академиков М. М. Шемьякина и Ю. А. Овчинникова, Москва, Россия

<sup>3</sup> Тихоокеанский государственный медицинский университет, Владивосток, Россия

<sup>4</sup> Российский национальный исследовательский медицинский университет имени Н. И. Пирогова, Москва, Россия

Морские полихеты *Chaetopterus varioopedatus* (Renier) (семейство *Chaetopteridae*) — космополиты, представляющие собой видовой комплекс из отдельных популяций-подвидов. При раздражении черви выпускают светящиеся (460 нм) облака слизи, при этом часто ярко светятся и их параподии. На сегодняшний день по-прежнему не ясно, как именно работает биолюминесцентная система этих полихет. Ранее было выдвинуто предположение, что люцифераза *C. varioopedatus* может быть использована для детекции ферроптоза — недавно открытого пути программируемой клеточной гибели, вызванной накоплением ионов двухвалентного железа. Целью исследования было выделить и охарактеризовать люциферазы *C. varioopedatus*, а также сравнить люциферазы *C. varioopedatus* из разных популяций. При выделении ответственного за биолюминесценцию фермента из замороженных образцов бразильских *C. varioopedatus* по усовершенствованной методике были получены две активные люциферазы — L1 и L2. Предположив, что одна из указанных люцифераз определяет свечение слизи, а другая — свечение параподий червей, эту же методику применили к раздельным образцам слизи и параподий живых дальневосточных *C. varioopedatus*. Однако их слизь оказалась несветящейся. Показано, что функцию свечения параподий полихет *C. varioopedatus* обеспечивает люцифераза L2, так как она обнаружена в общей биомассе бразильских полихет и в параподиях дальневосточных полихет. Свечение слизи бразильских *C. varioopedatus* обусловлено функционированием люциферазы L1, которая отсутствует в слизи дальневосточного подвида. Набор изоформ люцифераз полихет *C. varioopedatus* зависит от места их обитания.

**Ключевые слова:** биолюминесценция, люцифераза, полихеты, *Chaetopterus varioopedatus*, морские черви

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✉ **Для корреспонденции:** Александр Сергеевич Щеглов  
ул. Миклухо-Маклая, д. 16/10, г. Москва, 117997, Россия; jukart@mail.ru

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*Chaetopterus variopedatus* (Renier) belongs to the family *Chaetopteridae*, one of the most differentiated family of marine polychaetes inhabiting the benthic sediments during the adult stage, except for the uncommon pelagic species *C. pugaporcinus* (Osborn) [1]. Various researchers describe *C. variopedatus* as a species complex consisting of distinct populations/subspecies [2–4]. *C. variopedatus* is a cosmopolitan species living in temperate and tropical regions across the world. Various *C. variopedatus* subspecies are found in the coastal zones in Russia, Japan, Australia, Brazil, Europe and the USA [5]. A few years ago, phylogenetic relationships within *Chaetopteridae* were revised [1, 6], however, there remains considerable uncertainty about *C. variopedatus* sensu lato (Hartman).

*C. variopedatus* lives, hiding in the self-constructed U-shaped parchment-like tube, which is buried in the substrate. It has a segmented body with paired appendages, termed parapodia. *C. variopedatus* releases the blue-glowing ( $\lambda_{\text{max}}$  460 nm) clouds of mucus in response to aggressive external stimuli [7]. Concurrently, parapodia of all body segments also glow brightly. This phenomenon historically attracted the attention of curious observers, which is confirmed by numerous reports. Many researchers have tried to understand the biochemical aspects of the *C. variopedatus* luminescence over the past 70 years [8–10]. However, the results are still controversial. To date, it is yet unclear how exactly the bioluminescence system of these polychaetes functions.

As previously reported, preparation of luciferase, which was used to reproduce the characteristic bioluminescence reaction *in vitro*, was obtained from the *C. variopedatus* biomass, collected in the São Sebastião Strait off the coast of Brazil. In order to achieve *in vitro* bioluminescence, the alcoholic extract of *C. variopedatus*, containing the main substrate (luciferin) and the ferrous ions, was added to the preparation of luciferase [11].

We have suggested that the *C. variopedatus* luciferase may be used to detect ferroptosis [12], the recently discovered pathway of programmed cell death, resulting from accumulation of the ferrous ions [13]. Ferroptosis investigations are important for both fundamental and applied biomedical science. It has been shown that the processes similar to ferroptosis occur in some neurodegenerative diseases [14]. Moreover, ferroptosis inducers have a high potential as anticancer drugs [15].

Thus, detailed characterization and decoding of the *C. variopedatus* bioluminescence system is considered an urgent scientific challenge. This study was aimed at the extraction and characterization of *C. variopedatus* luciferases, and at comparative examination of luciferases, obtained from *C. variopedatus* of different populations.

## METHODS

### Collection of *C. variopedatus* biomass

*C. variopedatus* was collected at two locations: São Sebastião Strait of the coast of Brazil and Trinity bay in the Possiet Gulf of Japan Sea. The worms were pulled out of their tubes and immediately frozen in liquid nitrogen. The frozen polychaetes were shipped on dry ice and stored at  $-70^{\circ}\text{C}$ .

In order to obtain the glowing mucus, the worms were pulled out of the tubes, placed in the seawater and subjected to mechanical stimulation in the dark. The glowing mucus was collected with a pipette and frozen in liquid nitrogen.

### Luciferase extraction

A total of 100 g of the frozen *C. variopedatus* biomass were homogenized in 900 mL of 50 mM Tris buffer, pH 7.5. The

homogenate was sonicated using Ultrasonic Disintegrator UD-20 (Techpan; Poland) 5 times for 2 min on ice and subsequently centrifuged (25 000 g x 20 min) at  $4^{\circ}\text{C}$ . Supernatant was supplemented with ammonium sulfate to the final concentration of 500 mM and passed through a cellulose DEAE column (Cellulose DEAE-32, Serva; Germany), equilibrated with 500 mM ammonium sulfate. The resulting filtrate was loaded onto the 25 x 100 mm Phenyl Sepharose CL-4B column (Cytiva; USA), equilibrated with 500 mM ammonium sulfate. Luciferase was eluted with 5 mM Tris-HCl buffer, pH 7.5.

Fractions, possessing luciferase activity, were combined and loaded onto the 16 x 200 mm Sepharose DEAE FF column (Cytiva; USA), equilibrated with 20 mM Tris-HCl, pH 7.5; the column was washed with the same buffer, and the linear gradient elution was performed. Buffer A: 20 mM Tris-HCl, pH 7.5; buffer B: 500 mM NaCl, 20 mM Tris-HCl, pH 7.5. The flow rate was 4 mL/min, and the time of gradient was 25 min.

The resulting preparation was concentrated on the 10 kDa cell (Amicon; Ireland) and loaded onto the 26 x 400 mm Sephacryl S200 column (Cytiva; USA), equilibrated with 200 mM NaCl and 20 mM Tris-HCl, pH 7.5. Elution was performed with the same buffer at a rate of 1.5 mL/min. The pooled fractions possessing luciferase activity were supplemented with ammonium sulfate to the final concentration of 500 mM, the resulting solution was loaded onto the 5 x 90 mm C8 column (Cytiva; USA). The linear gradient elution was carried out. Buffer A: 500 mM ammonium sulfate, 5 mM Tris-HCl, pH 7.5; buffer B: 5 mM Tris-HCl, pH 7.5. The flow rate was 0.5 mL/min, and the time of gradient was 80 min.

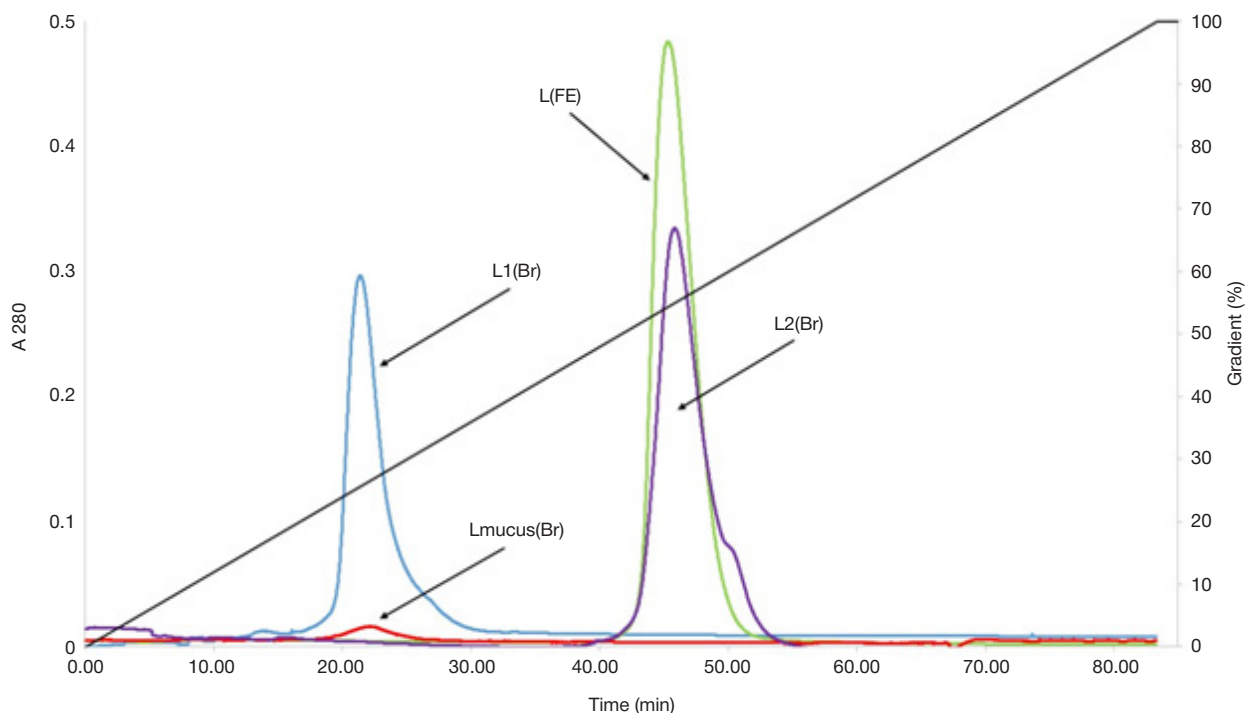
The resulting luciferase preparation was diluted twice with distilled water and loaded onto the 3 x 50 mm monoQ column (Cytiva; USA), equilibrated with 20 mM Tris-HCl, pH 7.5; the column was washed with the same buffer, and the linear gradient elution was performed. Buffer A: 20 mM Tris-HCl, pH 7.5; buffer B: 500 mM NaCl, 20 mM Tris-HCl, pH 7.5. The flow rate was 0.5 mL/min, and the time of gradient was 80 min.

The preparation was concentrated to a volume of 200  $\mu\text{L}$  on the 10 kDa centrifuge filter (Amicon; Ireland) with subsequent gel filtration through the 10 x 300 mm Superdex 200 column (Cytiva; USA), equilibrated with 100 mM NaCl, 50 mM Tris-HCl, pH 7.5. Elution was performed with the same buffer at a rate of 0.8 mL/min.

Luciferase was extracted from the glowing mucus (10 mL) by the same method. Luciferase activity was measured in accordance with the previously developed protocol [11].

The species specificity of the available *Chaetopterus* specimens was defined using the following primers: HC02198 (5'-TAAACTTCAGGGTGACCAAAAATCA-3') and LCO1490 (5'-TCAACAAATCATAAAGATATTGG-3'). DNA was extracted from each *C. variopedatus* frozen tissue sample with the use of the ExtractDNA Blood & Cells kit (Evrogen; Russia). After that the COI gene fragment sequences with a length of 650 bps were amplified using the listed above primers, and DNA extracted from each frozen tissue specimen was used as a template. PCR products were used for Sanger sequencing.

Mass spectrometry analysis of purified luciferase preparations, obtained from Brazilian and Far Eastern *C. variopedatus*, was carried out using the Q Exactive Plus mass spectrometer (Thermo Fisher Scientific; USA) coupled with the Ultimate 3000 Nano LC System (Thermo Fisher Scientific; USA) by means of the nanoelectrospray ionization source (Thermo Fisher Scientific; USA). Full scan MS acquisition was carried out with the following parameters: 140k resolution, normal mass range (500–2000 amu), AGC target  $3 \times 10^6$ , max. injection time



**Fig. 1.** Retention time values obtained for different *C. variopedatus* luciferases by chromatography involving the use of the monoQ anion exchange column, linear gradient; Br — Brazilian polychaetes, FE — Far Eastern polychaetes

30 ms. The raw data obtained were visualized with the XCalibur software (Thermo Fisher Scientific; USA).

## RESULTS

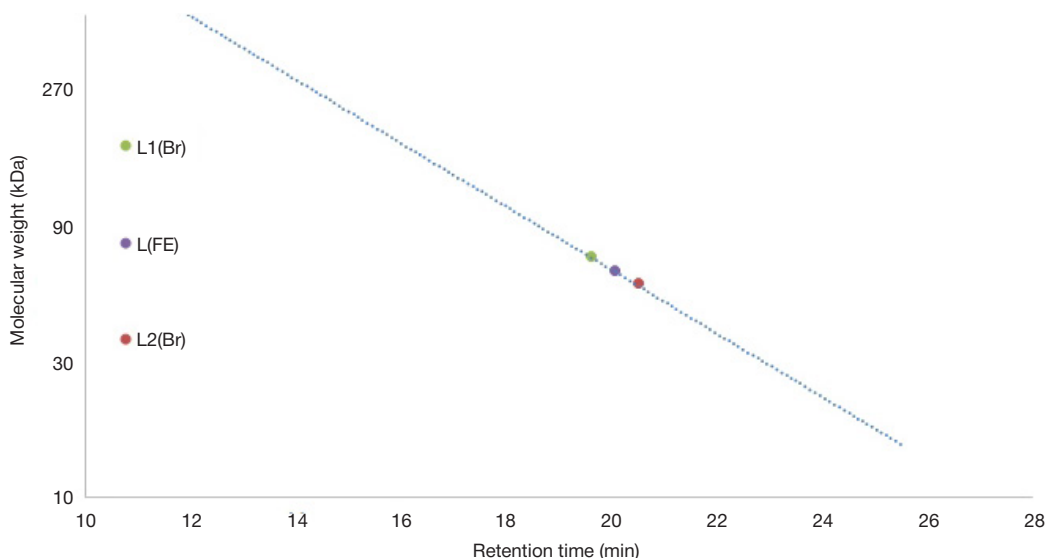
The use of high-resolution anion exchange chromatography resulted in the target preparation separated into two almost homogeneous luciferases: L1, retention time 21.5 min, and L2, retention time 46 min (Fig. 1). The native molecular weight of those, calculated based on the results of gel filtration, was 70 kDa and 60 kDa, respectively (Fig. 2).

Under denaturing conditions of SDS electrophoresis, luciferase L1 consisted of two mono subunits, each about 18 kDa, and L2 consisted of at least two different subunits with a mass of about 18 and 15 kDa (Fig. 3).

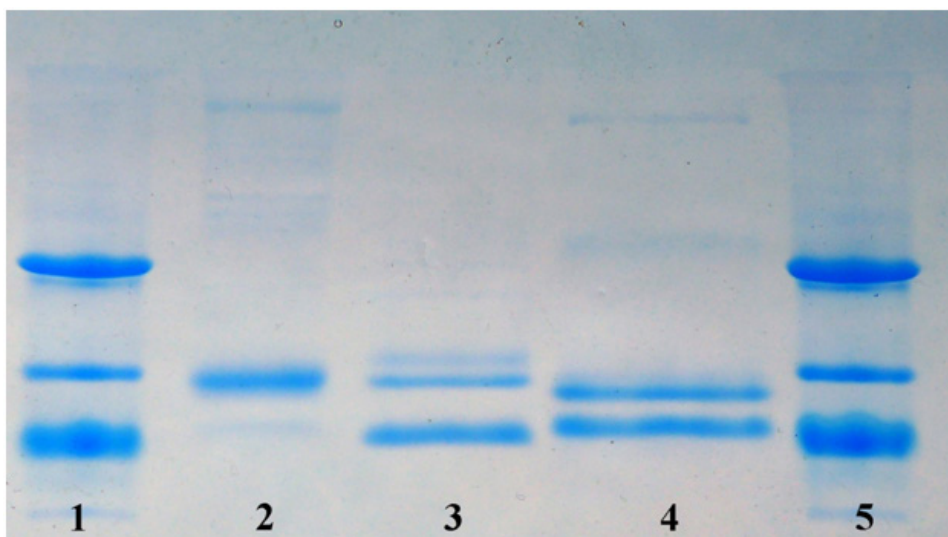
The existence of two different luciferases in one animal may be indicative either of their differing origins or of their specific

functions [16, 17]. Although the *C. variopedatus* luminescence is monochrome (blue), each of the worm's luciferases probably functions locally: for example, one could be responsible for parapodia luminescence, and the other could be responsible for luminescence of mucus, released by the worm into the external environment.

To test this hypothesis, we used the sample of mucus, collected and frozen separately when catching Brazilian *C. variopedatus*. Chromatographic analysis of the preparation, purified in accordance with the described above method, revealed the presence of one luciferase only, and the retention time on the monoQ column (45.5 min) for this luciferase showed reasonable agreement with the retention time measured for L1, previously extracted from the total worm biomass (see Fig. 1). No specific samples of the Brazilian polychaetes were available, and the amount of the obtained L1 and L2 turned out to be insufficient for sequencing. That is why the authors



**Fig. 2.** Molecular weight of different *C. variopedatus* luciferases defined by gel filtration; Br — Brazilian polychaetes, FE — Far Eastern polychaetes



**Fig. 3.** Denaturing electrophoresis (15% SDS-PAGE) of the preparations L1 (2) and L2 (3) obtained from Brazilian *C. variopedatus* and luciferase obtained from Far Eastern *C. variopedatus* (4). Tracks 1 and 5 — protein calibration mixture for SDS-PAGE electrophoresis: 29,000, 21,000, 12,500, 6,500 kDa

turned to the closer source of these worms, the Far East coast of Russia.

Polychaetes, collected in the Trinity bay in the Gulf of Possiet of Japan Sea, were initially identified as *C. variopedatus* based on morphological features and delivered to the laboratory alive. In order to better define the species specificity of the available *Chaetopterus*, the authors performed sequencing of the 650 bp fragment of the cytochrome C oxidase subunit I gene (COI). Sequence analysis using the GenBank database showed that the COI fragments of Brazilian and Far Eastern samples were highly homologous (over 99%) to those of two different subspecies: *C. variopedatus* (AM503096.1) and *C. cautus* Marenzeller (LC533809.1), respectively. To date, these subspecies have been merged into one species complex, *C. variopedatus* [6].

When collecting the Far Eastern polychaetes, their mucus was frozen separately. We failed to detect luminescence after defrosting the mucus at the laboratory. The portions of fresh mucus, obtained by mechanical and chemical stimulation of living worms, did not glow as well. Adding luciferin and ferrous iron to the mucus samples also did not lead to light emission. The efforts to extract luciferase from the mucus samples in accordance with the described above method were unsuccessful.

No problems with the detection of the Far Eastern polychaete parapodia luminescence were encountered. From those we managed to extract a highly purified luciferase L(FE)

with a native molecular weight of 65 kDa (see Fig. 2). The retention time on the monoQ column for this luciferase was 45.5 min, which was almost equal with the retention time of the luciferase L2 (see Fig. 1). Denaturing SDS electrophoresis demonstrates that both L(FE) and L2 of Brazilian polychaetes consist of two different subunits' molecular masses are also almost similar (see Fig. 3).

We performed mass spectrometry analysis of the preparations of purified luciferases, obtained from Brazilian and Far Eastern *Chaetopterus*. The results, mass-to-charge ratio ( $m/z$ ), charge ( $z$ ) and calculated molecular weight ( $m$ ), are presented in the Table.

Preparations of luciferases L2(Br) and L(FE) are significantly more heterogeneous compared to the L1(Br) preparation, which is probably due to posttranslational modifications and terminal amino acid cleavage. Mass sets, obtained for L2(Br) and L(FE), are almost the same, which attests to the enzymes similarity, and differ significantly from those obtained for L1(Br).

## DISCUSSION

Bioluminescence systems are widely implemented in various biomedical technologies [18, 19]. Luminescence bioimaging, the intravital imaging of cells and intracellular processes, is one of the important areas [20]. In case of successful decoding, the *C. variopedatus* luciferase may conceivably be used for development of ferroptosis sensor [12]. We have optimized the

**Table.** Mass spectrometry analysis of the preparations of purified luciferases, obtained from Brazilian (Br) and Far Eastern (FE) *Chaetopterus*

L1(Br)			L2(Br)			L(FE)		
$m/z$	$z$	$m$	$m/z$	$z$	$m$	$m/z$	$z$	$m$
1355.5	12	16253	1352.49	11	14866.39	1352.67	11	14868.37
1364	12	16356	1364.5	11	14998.5	1355.5	12	16254
1374.94	11	15113.34	1365.94	11	15014.34	1364.09	12	16357
1383.58	11	15208.38	1373.31	11	15095.41	1366.13	11	15016.43
			1374.77	11	15111.47	1373.41	11	15096.51
			1383.76	11	15210.36	1374.95	11	15113.45
			1396.93	12	16751.16	1383.85	11	15211.35
			1405.35	12	16852.2	1396.94	12	16751.28
						1404.44	12	16853.28

**Note:**  $m/z$  — mass-to-charge ratio;  $z$  — charge;  $m$  — molecular weight.

previously developed method for the *C. variopedatus* luciferase extraction [11], thereby obtaining an almost pure preparation, suitable for mass spectrometry analysis.

Originally, *C. variopedatus* caught in Brazil were used for analysis, however, logistical challenges forced us to use *C. variopedatus* caught in Primorsky Krai in Russia. It is interesting that unlike Brazilian polychaetes, Far Eastern *C. variopedatus* did not produce any glowing mucus. Two different isoforms of luciferase (L1 and L2) were found in Brazilian *C. variopedatus*, while only one (L) was found in Far Eastern polychaetes. Mass spectrometry and chromatography showed that L2 and L were extremely likely and differed from L1. Presumably, the function of parapodia luminescence in *C. variopedatus* polychaetes is ensured by luciferase L2 (L), since this luciferase has been found in the total biomass of Brazilian polychaetes and parapodia of Far Eastern polychaetes.

Luminescence of mucus in Brazilian *C. variopedatus* is due to the functioning of luciferase L1, which is lacking in the mucus of the Far Eastern subspecies.

## CONCLUSIONS

The findings lead to the following conclusions: 1. the *C. variopedatus* polychaete parapodia luminescence is attributed to the function of luciferase L2, since this luciferase has been identified in the total biomass of Brazilian polychaetes as L2(Br) and in parapodia of Far Eastern polychaetes as L(FE) identical to L2(Br); 2. the Brazilian *C. variopedatus* mucus luminescence is ensured by luciferase L1, which is lacking in the mucus of the Far Eastern subspecies; 3. the range of luciferase isoforms in *C. variopedatus* polychaetes might correlate with their place of origin.

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