Supplementary material for: Lukashanets D.A., Hihiniak Y.H. & Miamin V.Y. 2022. The phenomenon of extremely high abundances of the *Prasiola crispa*-associated micrometazoans in East Antarctica. *Polar Research 41*. Correspondence: Dzmitry A. Lukashanets, Universiteto ave., 17, Klaipėda University, Marine Research Institute, Klaipeda, 92294, Lithuania. E-mail: lukashanets.dima@gmail.com.

Abbreviations

PCR: polymerase chain reaction analysis

Methods of estimating the nutrient concentration in the samples

To determine the content of sodium (Na), phosphorus (P) and potassium (K), the sample preparation was carried out on a Berghof MWS-2 ultrasonic mineralizer. The 0.3 g dried sample was placed in a DAP-60K cuvette to which 3 ml of concentrated nitric acid (produced by Fisher and used in atomic absorption spectrometry) was added. After that, the cuvette was placed in the mineralizer, and sample preparation was carried out according to the following procedure.

After mineralization, the samples were filtered and the resulting solution was brought to a volume of 30 ml by adding deionized water with a resistivity of ≥ 18 Mohm / cm.

Na, P and K content were measured by a Perkin Elmer ICP ELAN DRC-e inductively coupled plasma mass spectrometer.

The ash content was determined under the following scheme.

The crucibles with weighed portions of dry substrate samples were placed in a cold muffle furnace and thefurnace temperature gradually brought to 800 °C and kept at this temperature for 2 h. Crucibles with ash residue were cooled in an open, switched-off furnace, and then in a desiccator for 30 minutes. After that, they were weighed with an error of less than 0.001 g. Each subsequent weighing was carried out after ashing for 1 hour and cooling for 30 minutes.

The analysis was considered complete if the difference between the results of two subsequent weightings did not exceed 0.01 g.

The mass fraction of organic matter with recalculation in carbon was estimated by the formula

$$X = (100 – A)\times 0.5,$$

where X is the carbon mass fraction, % and A is the ash mass fraction (%).

A muffle furnace was used for ashing, and a desiccator was used for drying (at 105 °C, to constant weight).

Determination of total nitrogen was carried out by the CINAO method, using a SP-830 Plus spectrophotometer.

DNA sequencing methods

The samples for genetic studies were 2 ml plastic tubes with several individuals of one species placed in 96 % ethanol. All manipulations were performed in compliance with the requirements of sterility.

We used the laboratory facilities of the SPC of the NAS of Belarus for Bioresources (DNA isolation and amplification) and the Institute of Genetics of the NAS of Belarus (the amplicon quality determining, purification, sequencing preparing and DNA sequencing). The equipment included the following: Bio-Rad C1000 Touch Thermal Cycler (USA); Bio-Rad horizontal electrophoresis chamber (USA); Biosan dry block thermostat (Latvia); Applied Biosystems™ SimpliAmp™ Thermal Cycler (USA); Bio-Rad GelDoc XR system (USA); Applied Biosystems 3500 genetic analyser (USA). The images obtained after electrophoresis were processed using Quantity One 4.4 software (Bio-Rad, USA). Statistical processing of the obtained data was carried out using MEGA 7 software.

To prepare for molecular and genetic studies, appropriate literature was used (Morita et al. 1987; Avise 1994; Werle 1994; Kumar et al. 2016) and methodological recommendations drawn from articles (as indicated below).

DNA isolation

The procedure was carried out in accordance with the protocol by Kaya et al. protocol (2009), with minor changes:

Adding 180 µl buffer T1 and 25 µl proteinase K to samples (isolated and cleaned rotifer individuals), which had been previously precipitated in a centrifuge 11 000 x g for 5 min. Vortexing the mixture, then incubating at 56 ° C until complete material lysis. Vortexing periodically.

Adding 200 µl buffer B3, vortexing thoroughly and then incubating at 70 °C for 10 min. Vortexing the sample again.

Adding 210 µl ethanol (96–100%) and vortexing thoroughly.

Transferring the mixture to a NucleoSpin Tissue Column, centrifuging for 1 min at 11,000 x g. Removing collection tube with liquid content in it. Transferring the column to a new collection tube;

Adding 500 µL buffer BW. Centrifugation for 1 min. at 11 000 x g. Removing the liquid from the collection tube, replacing the column.

Adding 600 µl buffer B5, centrifugation for 1 min. at 11,000 x g. Removing the liquid from the collection tube, replacing the column.

Centrifugation for 1 min. at 11 000 x g.

Transferring the column to a new 1.5 ml tube and adding 60–100 µl buffer BE. Incubation at room temperature for 1 min and centrifugation for 1 min at 11 000 x g.

DNA amplification

DNA primers designed by Folmer et al. (1994) for amplification of mitochondrial Cytochrome C oxidase subunit I from diverse metazoan invertebrates were used (manufactured by PrimeTech, Belarus):

LCOI 1490 5'-GGTCAACAAATCATAAAGATATTGG-3 '
HCOI 2198 5'-TAAACTTCAGGGTGACCAAAAAATCA-3 '

Amplification was carried out in 25 µl reaction mixture containing 2.5 µl 10x buffer with $(NH_4)_2SO_4$, 2.5 µl 10x dNTPs mixture, 1.5 mM $MgCl_2$, 5 pmol each of primers LCOI and HCOI, 0.01 units of Taq polymerase and 2 µl DNA of the studied samples.

Temperature and time regime of PCR for the first subunit of cytochrome oxidase (COXI) with universal primers (LCOI and HCOI):

1. 94 °C 5 min.,
2. 94 °C 1 min., 50 °C 1 min., 72 °C 90 sec. (35 cycles),
3. 72 °C 7 min.

The PCR was performed in a C1000 Touch Thermal Cycler (Bio-Rad Laboratories, Inc.).

Determining the amplification products quality

At the first stage, a qualitative determination of the amplified fragments was carried out. Electrophoretic separation of DNA fragments was carried out in 2% agarose gel with ethidium bromide fluorescent dye added. The method of horizontal electrophoresis in 1xTAE buffer using an operating electric field strength of 7 V / cm was applied. The amplified fragments lengths were determined by comparison with a Low Range DNA Ladder standard ranging from 50 bp to 1 kb. The lengths obtained during the reaction of amplicons were 680 bp. Visualization of PCR products was performed using the Bio-Rad GelDoc XR system and the images were processed using Quantity One 4.4 software, as mentioned.

The amplified fragments quality was found to be suitable for sequencing. The number of accumulated fragments of mitochondrial genes turned out to be quite large; therefore, all the samples provided were diluted four times to prevent possible sequencing errors.

Enzymatic purification of PCR products

The next step was the processing of samples with enzymes (Exo 1 and FastAP) to remove nonspecific DNA fragments and to prepare samples for terminal PCR. Each sample was supplemented with 1.2 µl mixture of Exo1 (0.3 µl) and FastAP (0.9 µl) enzymes. Samples were incubated for 30 minutes at 37 °C and then 15 minutes at 80 °C.

Preparing samples for sequencing

Terminal PCR was performed using a SimpliAmp™ Thermal Cycler.

Supplementary Table S1.The composition of the reaction mixture.

Reagent	Amount for one sample
H_2O	3.6 µl
Buffer	1.6 µl

Terminator	0.8 µl
Primer F	0.5 µl

After that, 6.5 µl reaction mixture and 1.5 µl DNA sample were added to each tube.

Supplementary Table S2. The temperature regime of terminal amplification.

Step	T(°C)	Duration	Number
I	96	1 min	1
II: denaturation	96	10 sec	
II: annealing	55	5 sec	40
II: elongation	60	4 min.	
III	12	Up to ∞	1

After terminal amplification, the samples were cleaned and dried according to the following protocol:

Adding 30 µl 96% alcohol in each tube;
Adding 2 µl 5M acetic acid, vortexing;
Keeping mixture in a freezer for 30 min.;
Centrifugation for 10 min., decanting the supernatant;
Adding 130 µl 70 % ethanol;
Centrifugation for 10 min., taking the supernatant with a dispenser;
Placing opened tubes in a thermostat at 45 °C until the precipitate is completely dry.

The sequencing results were aligned using the MEGA7 programme and compared with deposited data using the BLAST instrument.

Supplementary Table S3. Bdelloidea species recorded in *Prasiola crispa*, and methods of identification.

Species	Site	Identification methods		
		First descriptions	Morphometry	COXI sequencing
Adineta cf. *emsliei*	Progress2	+	+	-
Adineta grandis	Progress1	+	+	+
Adineta cf. *vaga*	Vechernyaya3	+	+	-
Habrotrocha cf. *antarctica*	Progress2	+	+	-
Habrotrocha cf. *constricta*	Vechernyaya3	+	+	-
Philodina gregaria	Progress1	+	+	+

Species identification of the rotifers: results of first descriptions and morphometric analysis

Details of the identification for Antarctic species

For *A. emsliei* we observed that the head (HW) is wider than that described by Iakovenko et al. (2015), and this increases the HW / HL ratio (see Supplementary Table S4). A wider spur pseudosegment (SSW) and a narrower corona (CW) in comparison with the type variety were also noted in *H. antarctica*, which led to a difference in the proportions of SL / SSW and CW / HW. In contrast, the body dimensions and proportions of *A. grandis* fully overlap with those reported in the literature (Iakovenko et al. 2015).

Details of the identification for A. vaga and H. constricta

We based the identification of *A. vaga* on the following features that differentiated it from the morphologically similar *A. emslei*: (1) triangular spurs (not needle-like on bulb-like bases); (2) colourless body (not orange); (3) difference in proportions FW/FL and RW/RL (both foot and rump wider than that of *A. emsliei*); (4) five teeth in each rake.

Also, the supposed *A. vaga* had a considerably more bulky body than *A. vaga* from Europe and a shorter foot. Trophi length was unexpectedly large.

Although many '*H. constricta*' records in the Antarctic should be considered as *H. antarctica* (Iakovenko et al. 2015), we leaned toward an identification of *H. constricta for* many individuals in the Vechernyaya3 samples because of: (1) upper lip with neither a notch nor two lobes; (2) spur shape (without elongated narrow tips and slightly swollen middle part); (3) larger sizes; (4) wider foot and rump; (5) dental formula 8/8 or 9/9 (not 7/7 or 4+3/4+3).

Supplementary Table S4. Body dimensions (maxima – and minima) and proportions of the bdelloid rotifer species: comparison of the data obtained in the present study with the literature data.

Dimensions (μm)	*Adineta grandis*		*Adineta* cf. *emsliei*	*Adineta* cf. *vaga*		*Habrotrocha* cf. *antarctica*	*Habrotrocha* cf. *constricta*		*Philodina gregaria*
	Pr. st.[a]	I. 2015 [b]	Pr. st.	I. 2015	Pr. st.	Pr. st.	I. 2015	Pr. st.	Pr. st.
TL	480	304–505	228–384	206–352	377–410	228	225–299	280–424	391–496
BW	107	60–152	46–113	40–87	63–93	43	35–58	43–82	79–102
HL	64–71	57–92	42–62	32–61	58–61	26–36	29–36	36–50	74
HW	55–60	48–71	41–55	27–47	50–52	28–35	28–37	41–48	65
CW	-[c]	-	-	-	-	25	25–32	33–40	98
NL	68–75	43–118	50–54	34–72	54–76	39	37–54	54–84	60
RL	55–58	36–72	44	27–55	46–52	30	29–39	19–53	52–64
RW	46–48	27–64	39	23–48	46–54	29	27–37	31–57	43–51
FL	48–53	28–69	42	27–46	42–63	32	22–37	21–54	57–71

SL	13	7–14	6–11	6–9	7–10	8–10	6–9	7–10	12–15
SSW	16	9–17	10–12	8–12	11–15	16–19	8–15	13–16	22–27
RaL	21–29	23.2–31.0	11–20	14.7–19.3	19.7–20.9	17.9–19.5	14.5–19.3	16.8–22.7	29–36
Proportion (%)	*Adineta grandis*		*Adineta* cf. *emsliei*	*Adineta* cf. *vaga*		*Habrotrocha* cf. *antarctica*	*Habrotrocha* cf. *constricta*		*Philodina gregaria*
	Pr. st.	I.2015	Pr. st.	I. 2015	Pr. st.	Pr. st.	I. 2015	Pr. st.	Pr. st.
BW/TL	23	16–31	15–34	19–27	16–25	19	15–20	14–29	20–21
CW/HW	-	-	-	-	-	71–89	79–91	74–88	151
FL/TL	11	6–15	11	10–16	11–15	14	10–12	6–15	14–15
SL/SSW	81	60–98	59–92	60–94	60–73	50–52	55–74	43–65	44–68
HL/TL	13	13–19	14–17	13–18	15	11	No data	11–13	15
HW/HL	78–94	66–97	84–111	71–94	85–87	92–97	96–100	89–112	88
NL/TL	16	11–27	17	14–21	17–9	17	15–35	14–25	15
RL/TL	12	7–16	12	11–16	11–14	13	11–15	6–14	13
RW/RL	83	74–103	88	74–98	101–105	97	80–110	103–158	78–83
FW/FL	52	40–56	46	29–45	43–58	63	49–74	59–91	52–68

[a] Present study. [b] Iakovenko et al. 2015.
[c]*Adineta* species do not have a corona-like rotatory apparatus.

Species identification of the rotifers: results of the DNA sequencing

Supplementary Table S5. Species identification based on the comparison of the obtained sequences with the data deposited in GenBank (sequences KJ543584.2, KJ543583.2 and KJ543586.1) despite being pointed here as '*Adineta sp.*' refer to *Adineta grandis* (Iakovenko et al. 2015))

Sample identification number	GenBank identification number	Species
# 40	OK325599	*Philodina gregaria*
# 41	OK325600	*Adineta grandis*

48 MT584979 *Philodina gregaria*

Supplementary Table S6. Write a caption here.

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Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Philodina gregaria isolate FIPO5 PG4 cytochrome oxidase subunit I (COI) gene, partial cds; mitochon…	Philodina gregaria	1088	1088	100%	0.0	98.00%	661	gi|1809655625|MN422502.1
Philodina gregaria isolate Bd47_05 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	Philodina gregaria	1110	1110	100%	0.0	98.67%	657	gi|1743045107|KJ543691.2
Philodina gregaria isolate Bd47_07 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	Philodina gregaria	1104	1104	100%	0.0	98.50%	657	gi|1743045111|KJ543693.2
Philodina gregaria isolate Bd47_01 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	Philodina gregaria	1104	1104	100%	0.0	98.50%	657	gi|1743045105|KJ543687.2
Philodina gregaria isolate Bd47_03 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	Philodina gregaria	1104	1104	100%	0.0	98.50%	660	gi|659495667|KJ543689.1
Philodina gregaria isolate Bd47_06 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	Philodina gregaria	1098	1098	100%	0.0	98.34%	657	gi|1743045109|KJ543692.2
Philodina gregaria isolate Bd47_04 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	Philodina gregaria	1098	1098	100%	0.0	98.34%	660	gi|659495669|KJ543690.1
Philodina gregaria isolate Bd47_02 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	Philodina gregaria	1098	1098	100%	0.0	98.34%	660	gi|659495665|KJ543688.1
Philodina gregaria isolate TB6 PG11 cytochrome oxidase subunit I (COI) gene, partial cds; mitochond…	Philodina gregaria	1092	1092	100%	0.0	98.17%	661	gi|1809655629|MN422504.1

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Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Adineta sp. Bd2_04 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	Adineta sp. Bd2…	1005	1005	100%	0.0	99.64%	611	gi|1743045103|KJ543584.2
Adineta sp. Bd2_03 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	Adineta sp. Bd2…	1005	1005	100%	0.0	99.64%	605	gi|1743045101|KJ543583.2
Adineta sp. Bd2_06 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	Adineta sp. Bd2…	1005	1005	100%	0.0	99.64%	621	gi|659495461|KJ543586.1
Adineta grandis isolate CBM1M_AG1 cytochrome c oxidase subunit 1 gene, partial cds; mitochondrial	Adineta grandis	1002	1002	99%	0.0	99.64%	595	gi|946831629|KP869899.1
Adineta grandis isolate FIPO5 AG2 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	Adineta grandis	1002	1002	100%	0.0	99.45%	661	gi|1807556099|MN367012.1
Adineta grandis isolate CzM3CY_AG4 cytochrome c oxidase subunit 1 gene, partial cds; mitochondrial	Adineta grandis	1000	1000	100%	0.0	99.45%	622	gi|946831664|KP869934.1
Adineta grandis isolate CzM2CY_AG5 cytochrome c oxidase subunit 1 gene, partial cds; mitochondrial	Adineta grandis	1000	1000	100%	0.0	99.45%	620	gi|946831660|KP869930.1
Adineta grandis isolate CzM2CY_AG3 cytochrome c oxidase subunit 1 gene, partial cds; mitochondrial	Adineta grandis	1000	1000	100%	0.0	99.45%	612	gi|946831658|KP869928.1
Adineta grandis isolate DAN149 AG4 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	Adineta grandis	1000	1000	100%	0.0	99.45%	578	gi|1807556139|MN367032.1

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Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Philodina gregaria isolate Bd47_01 cytochrome oxidase subunit I (COI) gene, partial cds; m…	Philodina gre…	1099	1099	100%	0.0	99.67%	657	gi|1743045105|KJ543687.2
Philodina gregaria isolate Bd47_02 cytochrome oxidase subunit I (COI) gene, partial cds; m…	Philodina gre…	1105	1105	100%	0.0	99.83%	660	gi|659495665|KJ543688.1
Philodina gregaria isolate Bd47_03 cytochrome oxidase subunit I (COI) gene, partial cds; m…	Philodina gre…	1110	1110	100%	0.0	100.00%	660	gi|659495667|KJ543689.1
Philodina gregaria isolate Bd47_04 cytochrome oxidase subunit I (COI) gene, partial cds; m…	Philodina gre…	1105	1105	100%	0.0	99.83%	660	gi|659495669|KJ543690.1
Philodina gregaria isolate Bd47_05 cytochrome oxidase subunit I (COI) gene, partial cds; m…	Philodina gre…	1105	1105	100%	0.0	99.83%	657	gi|1743045107|KJ543691.2
Philodina gregaria isolate Bd47_06 cytochrome oxidase subunit I (COI) gene, partial cds; m…	Philodina gre…	1105	1105	100%	0.0	99.83%	657	gi|1743045109|KJ543692.2
Philodina gregaria isolate Bd47_07 cytochrome oxidase subunit I (COI) gene, partial cds; m…	Philodina gre…	1099	1099	100%	0.0	99.67%	657	gi|1743045111|KJ543693.2
Philodina gregaria isolate DAN102 PG5 cytochrome oxidase subunit I (COI) gene, partial cd…	Philodina gre…	1094	1094	100%	0.0	99.50%	661	gi|1807556424|MN367783.1
Philodina gregaria isolate DAN102 PG6 cytochrome oxidase subunit I (COI) gene, partial cd…	Philodina gre…	1094	1094	100%	0.0	99.50%	661	gi|1807556426|MN367784.1