Four new choanoflagellate species from extreme saline environments: Indication for isolation-driven speciation exemplified by highly adapted Craspedida from salt flats in the Atacama Desert (Northern Chile)

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Received 25 June 2018; received in revised form 6 August 2018; accepted 8 August 2018
Available online 31 August 2018

Abstract

With this study we aim to extend the knowledge on the biogeography of craspedid choanoflagellates with additional data from extreme environments. Up to now, very little is known about choanoflagellates from extreme saline environments, as most studies have focused on marine and freshwater habitats. Though previously investigated high saline ice biota communities have indicated a possible adaptation to environments with high salt concentrations. Hypersaline endorheic basins, so-called salt flats or salares from the Atacama Desert in Northern Chile provide an intense environment regarding fluctuating and extreme salinities, which allow for studies on evolutionary adaptations of protists to hypersaline conditions. This study focused on choanoflagellate species isolated from different salt flats, their morphological characteristics using light and electron microscopy, molecular marker genes (SSU and LSU rDNA) and their salinity tolerance. Here, we described four new craspedid choanoflagellate species, highly adapted to the hypersaline environment of the Atacama Desert. This study extends our knowledge on choanoflagellate phylogeny and ecology and can become the basis for further molecular studies to understand the mechanisms of adaptations. Additionally, we emphasize the need of adding additional data such as autecological characteristics to amend species definitions, which is only possible from cultivated strains. This data would support the use of molecular data originating from metagenomic analyses also in an ecological context.

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Keywords: Adaptation; Atacama Desert; Craspedida; Choanoflagellatea; Phylogeny; Salinity tolerance

Introduction

The Atacama Desert in Northern Chile is one of the driest places on earth resulting in extreme environmental conditions, which demand a high degree of specialisation and adaptation from the residentiary unicellular eukaryotic organisms. Low precipitation and high temperatures have led to the formation of hypersaline endorheic basins within the desert. These salt flats are characterised by high variability regarding salinity - from low saline (less than 15 practical salinity units (PSU)) to almost salt-saturated waters (Risacher et al., 1999). Varying abiotic conditions, as seasonal fluctuations of chemical and physical water composition (e.g. nutrients, temperature, salinity), are challenging for the biota and affect species assemblages and ecosystem stability (Márquez-García et al., 2009). In addition, high doses of UV radiation, toxins like arsenic and heavy metals and an anomaly of salt composition, significantly differing from marine conditions, demand multiple adaptations. Recent gene-fingerprinting studies have shown high ecolog-
ical diversity and novelty of protists in hypersaline inland lakes, especially for choanoflagellates (Casamayor et al., 2013; Triadó-Margarit and Casamayor, 2013).

Choanoflagellates have great ecological impact on microbial food webs as they are highly efficient filter feeders, forming occasionally up to 40% of the biomass of heterotrophic flagellates in aquatic ecosystems (Arndt et al., 2000; Boenigk and Arndt, 2002; Buck and Garrison, 1988), living sedentary or freely suspended in the water column. The morphology among choanoflagellates is highly conserved, characterised by a cell with a single flagellum surrounded by a collar of microvilli (James-Clark, 1867; Leadbeater, 2015). Traditional taxonomy based on morphological characteristics of the external cell covering has proved to be misleading regarding a monophyletic classification within the Craspedida. Due to lack of data from the type specimen Salpingoeca gracilis (James-Clark, 1867), recent higher level taxonomic revision including molecular techniques, has led to the amalgamation of the former Codonosigidae and Salpingoecidae to only one family, the Salpingoecidae sensu Nitsche et al. (2011).

Here, we focus on the taxonomy, morphology and autecology of four new craspedid choanoflagellate species highly adapted to the abiotic conditions of the salt flats from the Atacama Desert. We aim to extend the knowledge on biogeography and taxonomic resolution of choanoflagellates by including new craspedid species from this extreme saline habitat and to prepare the ground for future detailed genetic analyses of adaptive processes based on comparative transcriptome analyses.

**Material and Methods**

**Sampling and cultivation**

Water samples were collected March 2017 from different salt flats (salares) of the Atacama Desert in Northern Chile (Table 1). Aliquots were cultured in 50 ml culture flasks (Falcon, Durham, USA) in artificial seawater (Instant Ocean, Aquarium Systems, Strasbourg, France) with salinities according to the original conditions. The cultures were enriched with the bacterial strain Pseudomonas putida (supplemented with cereal grass as carbon source) as food source for heterotrophic flagellates and regularly monitored by light microscopy (Zeiss Axiovert S 100) for choanoflagellate species. Mixed cultures containing choanoflagellates were diluted using the liquid aliquot method (LAM) (Butler and Rogerson, 1995).

**Video microscopy**

Morphology was investigated by an inverted light microscope system as described by Jeuck et al. (2014). Clonal cultures were transferred to modified Petri dishes (coverslips as base to optimize the optics) and observed using a Zeiss Axio Observer with a 100×/1.4 NA oil immersion objective (differential interference contrast (DIC)) and a water immersion condenser. Videos were taken using a black/white analogous Hamamatsu C6489 camera with noise reduction and contrast enhancement by the Allen Video Enhanced Contrast (AVEC) system (Hamamatsu, Argus-20) (for a detailed illustration see Stoupin et al. (2012)).

Videos were analysed frame by frame with VirtualDub (www.virtualdub.org) and processed with ImageJ (Abrámoff et al., 2004). Cell measurements were performed using the imaging software AxioVision (Zeiss).

**Electron microscopy**

For scanning electron microscopy (basic method see Nitsche (2016)), all samples were fixed in 2.5% cacodylate buffered glutaraldehyde (final concentration) at 4 °C for 120 min. Cultures remained in culture flasks for preparation. After the treatment with 1% osmium tetroxide for 10 min, samples were dehydrated in an ascending ethanol series of 30%, 50%, 70%, 80%, 90% and 96%. Samples were washed twice with the corresponding ethanol concentration.
and finally remained for 10 min in each solution. As a replacement for critical point drying, a final dehydration using 50:50 hexamethyldisilazane (HMDS)-ethanol solution was applied for 10 min followed by two washing steps with pure HMDS and 5 min incubation. Finally, the samples were allowed to dry. The bottom of each flask was cut out and stuck to a sample holder. Mounted samples were sputter coated with a 120 Å layer of gold and examined by SEM (FEI Quanta 250 FEG). SEM pictures were analysed for total flagellar length using the program MeasureMaster v.4.103.

Molecular biology

For the molecular analysis of the SSU and LSU rDNA, single cells were isolated using a micromanipulator (Patchman NP2 Eppendorf, Hamburg, Germany), transferred to 10 μl ddH2O and kept frozen at −20°C until PCR treatment. The SSU rDNA fragments were amplified with the primer pair 42F (5′-CTT AAR GAY TAA GCC ATG CA-3′) and 18S-Rev-1 (5′-ACC AAC GGG TTC GTC CGT-3) at a concentration of 0.1 μM, using a PCR Mastermix (2×) (VWR Life Science, Red Taq DNA Polymerase, Hassrode, Belgium). The PCR program started with a denaturation step at 98°C for 2 min, followed by 34 cycles of 96°C for 30 s, 52°C for 30 s (due to primers) and 72°C for 2.4 min and a final extension step by 11.11 min at 72°C. A 5 μl subsample of the first PCR was used as template for a reamplification (same PCR program) with the primer pair 82F (5′-GAA ACT GCG AAT GGC GTA GTG TGT ACA A-3′) and NLF1105/22 (5′-MRG GCT KAA TCT CAR YRG AGT TAT ACC G-3′) at a concentration 0.1 μM. The PCR program started with a denaturation step at 98°C for 2 min, followed by 30 cycles of 96°C for 30 s, 52°C for 30 s, 54°C for 30 s (due to primers) and 72°C for 4.2 min, finished by 11.11 min at 72°C. A 5 μl subsample of the first PCR was used as template for a reamplification with the primer pair NLF184/21 (5′-ACC CGC TGA AYT TAA GCA TAT C-3′) and NLR2098/24 (5′-AGC CAA TTC TWT TCC CGA AGT TAC-3′) at a PCR program with a denaturation step at 96°C for 5.01 min, followed by 34 cycles of 96°C for 30 s, 52°C for 30 s, 55°C for 30 s (due to primers) and 72°C for 2.4 min and a final extension step by 7.21 min at 72°C.

All PCR products were purified using the PCR Purification Kit (Bioscience, Jena, Germany). The sequencing of rDNA amplicons was done using the primers 82F+1630R+590F (5′-CGG TAA TTC CAG CTC CAA TAG C-3′)+600 R (5′-GCT ATT GGA GCT GGA ATT ACC G-3′)+1300R (5′-CAC CAA CTA AGA ACG GCC ATG C-3′) for re-amplified single cell SSU rDNA and NLF184/21+NLR2098/24+NLR1126R/22 (5′-CCG AAG TTT CCC TCA GGA TAG C-3′)+NLR1126R/22 (5′-GCT ATC CTG AGG GAA ACT TCG G-3′) for the LSU rDNA. Sequences were assembled using BioEdit (Hall, 1999).

Phylogenetic analysis

A concatenated, six-gene (SSU and LSU rDNA, hsp90, tubA, EFL and EF-1α) 9769 bp (manually corrected) alignment (Carr et al., 2017) including latest published sequences (Nitsche, 2016; Nitsche et al., 2017) was used to analyse the phylogenetic position of the new sequences (accession numbers see Suppl. Table 1). Sequences were individually integrated to the alignment using BioEdit (Hall, 1999). The alignment was analysed using Maximum likelihood (ML) and Bayesian inference (BI) methods. ML analysis was computed by RaxML v.8.2.10 (Stamatakis, 2014) using the GAMMA+P-Invar Model of rate heterogeneity as suggested by the author with 1000 replicates for the bootstrap analysis. The Bayesian analysis was performed by MrBayes v.3.2.6 (Ronquist et al., 2012) running a GTR+I+Γ model and a four-category gamma distribution to correct for among site rate variation. The search consisted of two parallel chain sets run at default temperatures with a sample frequency of 10 and run until the average standard deviation of split frequencies dropped below 0.01. The analysis consisted of 1,750,000 generations, with a burnin of 437,500 before calculating posterior probabilities. The choanoflagellate phylogeny was rooted with a two-taxon ichthyosporean clade and an eight-taxon metazoan clade. The uncorrected pairwise distances (only SSU rDNA) were estimated using MEGA 7 (Kumar et al., 2016). The alignment is available from the author upon request.

Auteology

To investigate the auteology of the new species, salinity tolerance experiments were performed. The cultures remained at least for three weeks in artificial seawater with the most suitable salinity for culture growing (tested before) (“Salpingoeca” crinita sp. nov. and “Salpingoeca” surina sp. nov. at 90 PSU, “Salpingoeca” huasca sp. nov. and “Salpingoeca” prava sp. nov. at 35 PSU). A gradient of different salinities (0, 1, 3, 5, 12, 20, 35, 60, 90 and 150 PSU) with three replicates of each treatment and one control (culture conditions) was established in culture flasks at room temperature to test for species’ survival. Salinity was raised and decreased from culture conditions stepwise (as described above) after an acclimatisation of 48 h. The bacterial strain P. putida (enriched with cereal grass as carbon source) was added to each culture to exclude a food limitation due to the effect of salinity changes on the
environmental bacteria community. As tested in previous studies, this bacterial strain is suitable as food source for choanoflagellates and tolerant regarding changes in salinity (Nitsche, 2014). Culture flasks were monitored every 24 h with cell counts (MoxiZ, ORFLO, Ketchum, USA) for 5 days.

**Results**

In this study, we cultivated and described four new craspedid choanoflagellate species. The description is based on molecular data (SSU and LSU rDNA) (Fig. 1), a detailed morphological approach including light and electron microscopy.
Fig. 2. Morphology of “Salpingoeca” crinita sp. nov. and “Salpingoeca” prava sp. nov. (A–F) “Salpingoeca” crinita sp. nov.: (A) Schematic illustration of the specimen; (B) collared cell; (C) flagellum; (D) cell overview with stalk; (E) EM micrograph of whole cell; (F) enlargement of the flagellum showing small filaments in an opposite arrangement. (G–L) “Salpingoeca” prava sp. nov.: (G) Schematic illustration of collared specimen; (H) Schematic illustration of specimen in life cycle stage with pseudopodia; (I&J) collar bearing cells; (K) cell with pseudopodia; (L) EM micrograph of whole cell. Scalebar: 2 μm. Abbreviations: b — Bacterium; fl — Flagellum; m — Microvilli; n — Nucleus; p — Pseudopodium; st — Stalk; th — Theca.

(Fig. 2, Fig. 3), as well as autecological experiments regarding the salinity tolerance (Fig. 4). We were able to maintain all species as monocultures. All new species were denominated in quotation marks as “Salpingoeca” species, reflecting the uncertain taxonomy of this genus which is not monophyletic (Fig. 1). Until molecular data on the type species, Salpingoeca gracilis (James-Clark, 1867; Thomsen, 1977), is available, we refrained from generating artificial genera.
Fig. 3. Morphology of “Salpingoeca” surira sp. nov. and “Salpingoeca” huasca sp. nov. (A–G) “Salpingoeca” surira sp. nov.: (A) Schematic illustration of the specimen; (B) cell body with collar; (C) “fast swimmer” with shortened collar and pseudopodia; (D) EM micrograph of whole cell; (E) thecate cell with stalk; (F&G) cell with pseudopodia. (H–O) “Salpingoeca” huasca sp. nov.: (H) Schematic illustration of the specimen; (I) collared cell; (J) cell with pseudopodia; (K) “fast swimmer” with shortened collar; (L) theca; (M) collar; (N) EM cell overview; (O) “fast swimmer” with shortened collar. Scalebar: 2 μm. Abbreviations: b — Bacterium; fl — Flagellum; m — Microvilli; p — Pseudopodium; st — Stalk; th — Theca.
Fig. 4. Salinity tolerance ranges of all newly described species. Bars in dark grey indicate growth, bars in light grey the sporadic presence of species. Dots show the measured original salinity of the salt flat, average marine salinity given by dashed line.

to avoid future renaming problems. All new sequences were deposited in GenBank (Table 1) and the uncorrected pairwise distances of the SSU rDNA are available from the author upon request.

Taxonomy

Morphological data from light and scanning electron microscopy, e.g. the presence of an organic cell coating (Fig. 2, Fig. 3), support the allocation of the species “Salpingoeca” crinita sp. nov., “Salpingoeca” prava sp. nov., “Salpingoeca” surira sp. nov. and “Salpingoeca” huasca sp. nov. to the order of Craspedida (Carr et al., 2017; Nitsche et al., 2011).

Phylogenetic analysis

Partial SSU and LSU rDNA of the four species were sequenced (Table 1) and analysed in a concatenated phylogeny (Fig. 1), based on previous studies (Carr et al., 2017; Nitsche et al., 2011, 2017). The 60 taxa of choanoflagellates, 8 taxa of Metazoa and 2 taxa of Ichthyosporea formed distinct clades with strong support (1.00 biPP; 100% mlBP). As described previously, we found strong Bayesian inference support (1.00 biPP) for the orders Craspedida and Acanthoeucida to be monophyletic. Within the Craspedida we recorded a high support (1.00 biPP; 100% mlBP) that the newly introduced species “Salpingoeca” crinita sp. nov., “Salpingoeca” surira sp. nov. and “Salpingoeca” huasca sp. nov. do cluster together forming a new clade close to Salpingoeca rosetta (Fig. 1). Uncorrected pairwise distances revealed phylogenetic distances of 14.7–15.7% to Salpingoeca rosetta and 13.3–14.3% to Microstomoeca roanoka (only based on SSU rDNA). Our sequences of “Salpingoeca” prava sp. nov. extended the most distant branch within the Craspedida of Salpingoeca tuba and Salpingoeca doliolothecata with moderate support (0.71 biPP; <50% mlBP). Pairwise distances showed phylogenetic distances of 9.2% to Salpingoeca tuba and 13.9% to Salpingoeca doliolothecata.

Autecology

We were able to successfully cultivate all species in a broad range of salinities (Fig. 4). All species were able to tolerate the salinity decrease to low saline waters although originating from hypersaline habitats (Table 1). In particular, “Salpingoeca” crinita sp. nov. (original salinity: 100 PSU) showed positive growth from 12 to 150 PSU, “Salpingoeca” prava sp. nov. (original salinity: 40 PSU) from 12 to 90 PSU and “Salpingoeca” surira sp. nov. (original salinity: 50 PSU) as well as “Salpingoeca” huasca sp. nov. (original salinity: 288 PSU) from 5 to 150 PSU.

Descriptions of the new species

For taxonomic description, species were morphologically analysed regarding cell characteristics (measurements) and life cycle stages (holotype and paratype) (Table 2).

Order: Craspedida (Cavalier-Smith, 1997).
Family: Salpingoecidae (Saville-Kent, 1880 emend. sensu Nitsche et al. (2011)).
Genus: Salpingoeca.
Species: “Salpingoeca” crinita sp. nov. (Fig. 2A–F), type strain: Heterotrophic Flagellate Collection Cologne (HFCC) strain 1287.

Diagnosis: Hypersaline Salpingoeca-like species, about 4 × 3 μm in size with a tight-encircling theca. The species occurs as solitary cells attached to surfaces and
exhibits a long stalk (about 10 µm) and a flagellum up to 2.5–6 times longer than the protoplast. The flagellum is featured by the formation of small filaments in an opposite arrangement. Species can tolerate low saline to hypersaline conditions (12–150 PSU).

**Etymology:** *crinita* (Latin, adjective, feminine) from Latin “hairy” in reference to the observed flagellum with small filaments.

**Type locality:** Salar de Atacama, a salt flat in the Atacama Desert in Northern Chile (23°03′58.59″S 67°43′36.60″W) (Table 1).

**Holotype:** Specimen illustrated in Fig. 2A.

**Description:** *Salpingoeca*-like species with a body length of 3.6–5.0 µm (4.2 ± 0.4 µm on average, N = 22) and a body width of 2.6–4.1 µm (3.3 ± 0.4 µm on average, N = 22). The theca is close-encircling to the protoplast. The collar has a variable length of 1.0–9.0 µm (4.8 ± 2.1 µm on average, N = 17) and a width of 2.9–8.0 µm (5.4 ± 1.4 µm on average, N = 17). The flagellum has a length from 9.1–30.5 µm (18.9 ± 7.0 µm on average, N = 14). The stalk is 5.7–14.1 µm long (9.9 ± 2.5 µm on average, N = 14).

**Type sequence data:** The SSU rDNA sequence was deposited in GenBank database with the accession numbers MH490946 (SSU) and MH490947 (LSU). The closest relative sequence on NCBI nucleotide BLAST® results in an uncultured eukaryote marine clone (FN690480) with a maximum identity of 93%.

**Species:** “Salpingoeca” *prava* sp. nov. (Fig. 2G–H), type strain: HFCC strain 1244.

**Diagnosis:** Hypersaline *Salpingoeca*-like species, about 5 × 4 µm in size. Cells frequently occur in accumulations (colony-like) attached to surfaces with a stalk. Fast swimming cells with a shortened collar and pseudopodia present. Species can tolerate low saline to hypersaline conditions (5–150 PSU).

**Etymology:** *prava* (Latin, adjective, feminine) from Latin “bended” in reference to the body shape by light microscopic view.

**Type locality:** Salar de Talar, a salt flat in the Atacama Desert in Northern Chile (23°03′58.59″S 67°43′36.60″W) (Table 1).

**Holotype:** Specimen illustrated in Fig. 2G–H.

**Description:** *Salpingoeca*-like species with a body length of 3.1–4.6 µm (3.6 ± 0.4 µm on average, N = 27) and a body width of 1.9–3.1 µm (2.6 ± 0.3 µm on average, N = 27). The collar has a length of 1.7–5.2 µm (3.5 ± 1.3 µm on average, N = 11) and a width of 1.7–5.6 µm (3.2 ± 1.3 µm on average, N = 11). The flagellum has a length from 4.1–12.6 µm (7.9 ± 2.6 µm on average, N = 23).

**Type sequence data:** The SSU and LSU rDNA sequences were deposited in GenBank database with the accession numbers MH490946 (SSU) and MH490947 (LSU). The closest relative sequence on NCBI nucleotide BLAST® results in an uncultured eukaryote marine clone (FN690480) with a maximum identity of 93%.

**Species:** “Salpingoeca” *surira* sp. nov. (Fig. 3A–G), type strain: HFCC strain 1244.

**Diagnosis:** Hypersaline *Salpingoeca*-like species, about 3.5 × 2.5 µm in size with a protoplast almost filling the theca. Species present in both a collared life cycle stage and without a visible collar, but many pseudopodia. Solitary cells are strongly attached to surfaces. Species can tolerate low saline to hypersaline conditions (12–90 PSU).

**Etymology:** *surira* (adjective, feminine) in reference to the type locality.

**Type locality:** Salar de Surire, a salt flat in the Atacama Desert in Northern Chile (18°49′43.00″S 69°03′40.5″W) (Table 1).

**Holotype:** Specimen illustrated in Fig. 3A.

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**Table 2.** Morphometric data of the new species based on light microscopy. All measurements in µm, average N = 18, SD = Standard deviation.

<table>
<thead>
<tr>
<th>Species</th>
<th>Length cell body</th>
<th>Width cell body</th>
<th>Length flagellum</th>
<th>Length collar</th>
<th>Width collar</th>
<th>Length stalk</th>
</tr>
</thead>
<tbody>
<tr>
<td>“Salpingoeca” <em>crinita</em> sp. nov.</td>
<td>Mean: 4.2</td>
<td>3.3</td>
<td>18.9</td>
<td>4.8</td>
<td>5.4</td>
<td>9.9</td>
</tr>
<tr>
<td></td>
<td>SD: 0.4</td>
<td>0.4</td>
<td>7.0</td>
<td>2.1</td>
<td>1.4</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>Min.: 3.6</td>
<td>2.6</td>
<td>9.1</td>
<td>1.0</td>
<td>2.9</td>
<td>5.7</td>
</tr>
<tr>
<td></td>
<td>Max.: 5.0</td>
<td>4.1</td>
<td>30.5</td>
<td>9.0</td>
<td>8.0</td>
<td>14.1</td>
</tr>
<tr>
<td>“Salpingoeca” <em>prava</em> sp. nov.</td>
<td>Mean: 3.6</td>
<td>2.6</td>
<td>7.9</td>
<td>3.5</td>
<td>3.2</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>SD: 0.4</td>
<td>0.3</td>
<td>2.6</td>
<td>1.3</td>
<td>1.3</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Min.: 3.1</td>
<td>1.9</td>
<td>4.1</td>
<td>1.7</td>
<td>1.7</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Max.: 4.6</td>
<td>3.1</td>
<td>12.6</td>
<td>5.2</td>
<td>5.6</td>
<td>–</td>
</tr>
<tr>
<td>“Salpingoeca” <em>surira</em> sp. nov.</td>
<td>Mean: 4.9</td>
<td>3.8</td>
<td>12.9</td>
<td>4.3</td>
<td>4.1</td>
<td>6.4</td>
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<tr>
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<td>SD: 0.5</td>
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<td>4.8</td>
<td>2.1</td>
<td>1.6</td>
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</tr>
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<td>Max.: 5.7</td>
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<td>22.6</td>
<td>7.9</td>
<td>6.6</td>
<td>8.3</td>
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<tr>
<td>“Salpingoeca” <em>huasca</em> sp. nov.</td>
<td>Mean: 4.5</td>
<td>3.9</td>
<td>15.9</td>
<td>4.6</td>
<td>4.6</td>
<td>8.1</td>
</tr>
<tr>
<td></td>
<td>SD: 0.4</td>
<td>0.5</td>
<td>5.9</td>
<td>2.7</td>
<td>1.1</td>
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</tr>
<tr>
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<td>Min.: 3.7</td>
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<td></td>
<td>Max.: 5.4</td>
<td>5.4</td>
<td>24</td>
<td>9.3</td>
<td>6.7</td>
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</table>
Description: Salpingoeca-like species with a body length of 4.1–5.7 μm (4.9 ± 0.5 μm on average, N = 23) and a body width of 3.1–4.5 μm (3.8 ± 0.4 μm on average, N = 24). The collar has a variable length of 1.0–7.9 μm (4.3 ± 2.1 μm on average, N = 14) and a width of 1.9–6.6 μm (4.1 ± 1.6 μm on average, N = 14). The flagellum has a length from 6.1–22.6 μm (12.9 ± 4.8 μm on average, N = 15). The stalk is 4.4–8.3 μm long (6.4 ± 1.5 μm on average, N = 6).

Type sequence data: The SSU and LSU rDNA sequences were deposited in GenBank database with the accession numbers MH490948 (SSU) and MH490949 (LSU). The closest relative sequence on NCBI nucleotide BLAST® results in Salpingoeca rosetta (EU011941) with a maximum identity of 93%.

Species: “Salpingoeca” huasca sp. nov. (Fig. 3 H-O), type strain: HFCC strain 1286.

Diagnosis: Hypersaline Salpingoeca-like species, about 4.5 x 4 μm in size. Solitary cells attached to surfaces with a stalk present as well as fast swimming cells with a shortened collar. Species can tolerate low saline to hypersaline conditions (5–150 PSU).

Etymology: huasca (adjective, feminine) in reference to the type locality.

Type locality: Salar de Huasco, a salt flat in the Atacama Desert in Northern Chile (20°15’S57.5”S 68°49’16.0”W) (Table 1).

Holotype: Specimen illustrated in Fig. 3 H.

Description: Salpingoeca-like species with a body length of 3.7–5.4 μm (4.5 ± 0.4 μm on average, N = 21) and a body width of 3.0–5.4 μm (3.9 ± 0.5 μm on average, N = 23). The collar has a variable length of 0.7–9.3 μm (4.6 ± 2.7 μm on average, N = 15) and a width of 2.7–6.7 μm (4.6 ± 1.1 μm on average, N = 15). The flagellum has a length from 6.6–24 μm (15.9 ± 5.9 μm on average, N = 21). The stalk is 6.5–10.4 μm long (8.1 ± 1.9 μm on average, N = 9).

Type sequence data: The SSU rDNA sequence was deposited in GenBank database with the accession number MH490950. The closest relative sequence on NCBI nucleotide BLAST® results in Microstomoeca roanoka (HQ026770) with a maximum identity of 90%.

Discussion

The Atacama Desert provides a unique environment with challenging conditions as the different investigated salt flats in this study showed a broad range of hypersaline water bodies (Table 1) demanding specific adaptations from the residential organisms. In this study, we were able to describe four new craspedid species from these extreme saline environments. Our phylogenetic analysis points toward a high degree of adaptation as the new species clustered in distinct branches with high p-distances (9.2–15.7%) to all previous sequenced choanoflagellates. In addition, none of these sequences was previously recorded from high throughput sequencing (HTS) or environmental studies such as Tara Ocean project and others, indicating their geographic restriction. We hypothesise that at least two marine last common ancestors were transported independently to the different salt flats by birds (flamingos and other water birds migrating from ocean to the salt flats), evolving to the present species. The distinct geographical separation and isolation of the investigated salt flats will be the basis for future studies to further elucidate protist speciation in these isolated habitats.

Three of our described species, “Salpingoeca” crinita sp. nov., “Salpingoeca” huasca sp. nov. and “Salpingoeca” surira sp. nov. extended the clade around the model organism Salpingoeca rosetta (Fig. 1). “Salpingoeca” prava sp. nov. clustered with Salpingoeca tuba and Salpingoeca dolichothecata, forming a basal group within the Craspedida (Fig. 1). We found that just like in clade 1 and clade 2 (see Carr et al., 2017), there are no diagnostic morphological traits to define the phylogenetic clade 3, containing Salpingoeca tuba and Salpingoeca dolichothecata, which is now extended by “Salpingoeca” prava sp. nov. (Fig. 2). In addition to the phylogenetic analysis, our detailed morphological approach showed polymorphism in several species representing different life cycle stages. Therefore, we support the inclusion of all paratypes in an accurate species description to avoid future redundant descriptions (Jeuck et al., 2014; Stoupin et al., 2012). Scanning electron microscopy revealed small filaments attached to the flagellum of “Salpingoeca” crinita sp. nov. with yet unknown function (Fig. 2).

Aside with the recent taxonomic revision, studies suggest to amend morphological and molecular data with autecological traits such as habitat preferences and salinity tolerance to increase the accuracy and stability of species definition in an ecological context (Carr et al., 2017; Nitsche, 2014). As the abiotic environmental parameters play a key role for species adaptation and speciation (Atkins et al., 2002; Filker et al., 2013; Nitsche, 2014; Nitsche and Arndt, 2015), the salinity tolerance of all species, as they originate from salt flats with variable salinities, was tested. The autecological approach revealed that our newly described species can cope with a broad range of salinities, from low saline to hypersaline waters (Fig. 4) supporting a high adaptation to the original hypersaline environment. In general, the cultivation of choanoflagellates and other protists is mainly limited by the environmental bacterial diversity. It must be considered that an environmental bacterial community shift due to salinity changes might have favoured microbes of low food quality or species producing toxic metabolites and hence an indirect effect of the salinity shift might have occurred. To exclude a possible food limitation, we chose the bacterial strain P. putida as a supplemental food source. These bacteria were tested previously as a suitable food source for protists and are able to tolerate salinity changes (Nitsche, 2014). It should be mentioned that this bacterial strain may have posed a challenge for “Salpingoeca” prava sp. nov. as this species is probably dependent on smaller bacteria due to its small size (Table 2). To understand the abiotic condi-
tions of the investigated salt flats, the elementary composition was analysed via inductively coupled plasma mass spectrometry (MS platform, Biocenter Cologne, data not shown). This preliminary investigation showed intense chemical variations on salt structuring components but also the deposit of toxic elements, such as arsenic. As arsenic is a highly volatile component, more sophisticated ways of detection and quantification will be applied in cooperation with the Department of Geosciences, University of Cologne. As the adaptive mechanisms to extreme conditions, like hypersaline environments or the presence of arsenic, are well-studied for prokaryotes (Dorador et al., 2008; Kartal et al., 2006; Lara et al., 2012; Padan et al., 1989; Steil et al., 2003), further investigations on the highly adapted choanoflagellates described in this study, based on comparative transcriptomes, could give new insights for eukaryotic adaptation to extreme conditions and specially about horizontal gene transfer from prokaryotes to eukaryotes (Yue et al., 2013).

Acknowledgements

We are thankful for fruitful discussions with Cristina Dorador and Chris Harrod from the University of Antofagasta during the expedition in the Atacama Desert. Many thanks go to Nina Grella and Linda Müller who helped isolating the strains, the MS platform for using the ICP-MS (University of Cologne) and Michael Staubwasser for discussions on the chemical composition of salt flats. Special thanks to Martin Carr for helpful discussions and comments. This work was funded by the grants of the German Research Foundation (DFG) in the frame of CRC 1211: Earth — Evolution at the dry limit to H.A. (B03) and the SPP 1991: Taxon-omics — New Approaches for Discovering and Naming Biodiversity to F.N. (1097/3-1).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ejop.2018.08.001.

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