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# New salinity-tolerant species of *Gyrodactylus* (Platyhelminthes, Monogenea) on intertidal and supratidal fish species from the Chilean coast

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**Abstract**

**Purpose** The intertidal and supratidal coastal zone challenges the osmoregulatory capacity of its aquatic inhabitants. Four new species of *Gyrodactylus* ectoparasites on two intertidal fishes from Chile are described based on molecular and morphological analyses.

**Methods** Monogeneans were found on two fish species, the clingfish *Sicyases sanguineus* Müller & Troschel, 1843, and the combtooth blenny *Scartichthys viridis* Valenciennes, 1836. The morphology was described using drawings, and minimal measurements. The parasites were barcoded via the sequencing of the ribosomal DNA over ITS1 - 5.8S – ITS2.

**Results** The air-breathing clingfish *S. sanguineus* carried *Gyrodactylus amphibius* sp. nov., hiding in the ventral sucker formed by the modified pectoral fins of the fish. The intertidal combtooth blenny *S. viridis* carried three other new species: *Gyrodactylus scartichthi* sp. nov., *Gyrodactylus viridae* sp. nov., and *Gyrodactylus zietarae* sp. nov.

**Conclusion** The four new species were all phylogenetically related to the previously described *G. chileani* Zięta ra et al. 2012 on the triplefin *Helcogrammoides chilensis* Cancino, 1960 in the same habitat. Thus, the five Chilean Pacific *Gyrodactylus* species formed a statistically well supported (100 %) monophyletic clade together with three geographically distant species recorded in Europe. The Chilean Pacific parasites are not related to *G. salinae* and *G. magadiensis*, parasites described in extreme osmotic stress environments earlier.

**Keywords** *Gyrodactylus*, osmotic adaptation, marine dispersal, intertidal zone, ITS barcoding

## Introduction

The morphology of viviparous fish ectoparasites of the genus *Gyrodactylus* von Nordmann, 1832, is rather conservative, but the spectrum of ecophysiological adaptations is wide, as is the "invisible" DNA variation [1]. For the small and thin-skinned aquatic organisms, the maintenance of water balance and constant osmotic pressure is crucial for the adaptation to different environments and salinity gradients, much studied in fish [e.g., 2–4]. Malmberg [5] developed a subgenus division and systematics of *Gyrodactylus* based on the morphology of the osmoregulatory protonephridial system. Maintaining approximately the ocean water level of electrolyte concentration is the universal standard. In freshwater, extra water is continuously pumped out. According to Malmberg's systematics, two subgenera are freshwater bound: *G.* (*Limnonephrotus*) and *G.* (*Gyrodactylus*). In high salinity, osmotic drying is the challenge. The subgenera *G.* (*Neonephrotus*), *G.* (*Metanephrotus*), *G.* (*Mesonephrotus*) and *G.* (*Paranephrotus*) represented groups in marine environments, but the division was morphological (and phylogenetic), not physiological. The osmoregulatory system determines the fine details of ecology in the narrow but long and vulnerable tidal zone [6], studied, for example, among free-living nematodes [7]. Fish parasites cannot make the salinity orientation independently, but have to rely on their host [8].

In this paper, we describe four new species of *Gyrodactylus* living in an osmotically challenging environment on the Chilean rocky coast, utilizing the resolution of the sequencing of the ITS-5.8S-ITS2 nuclear ribosomal gene region. This allows a definite barcoding of the species, but also a rough phylogenetic comparison of the new species with other species known in extreme salinities. We may ask, whether the extreme salinity tolerant species are phylogenetically derived from the common root, or whether the tolerance evolved independently in separate branches of the phylogenetic tree.

We describe the first genuinely amphibious *Gyrodactylus* sp. on the amphibious clingfish *Sicyases sanguineus* Müller & Troschel 1843 from the Chilean coast [9]. Th evolution of amphibious behavior has occurred repeatedly in ecologically diverse fish families [10]. Gyrodactylid parasites are known in many of these fish families, but highly amphibious parasites have not been reported before, to our knowledge. In addition to the clingfish parasite, we also describe gyrodactylid parasites on *Scartichthys viridis* Valenciennes 1836, which was classified as a completely aquatic (not amphibious) blenny in the evolutionary study of Ord and Cooke [10].

## Material and methods

The collection occurred in Las Cruces, Central Chile (33.48333°S; 71.616667° W decimal degrees) [11]. Six small (< 3 cm) juvenile specimens of the clingfish *Sicyases sanguineus* Müller & Troschel 1843 (local name pejesapo) were caught on the tidal rocks and stored in strong ethanol as such, in 20zz. One specimen of the combtooth blenny *Scartichthys viridis* Valenciennes 1836 (local name borrachilla) was caught from tidal ponds in 20zz, the fins were cut and stored in ethanol. The fish were caught one by one, using a hand net.

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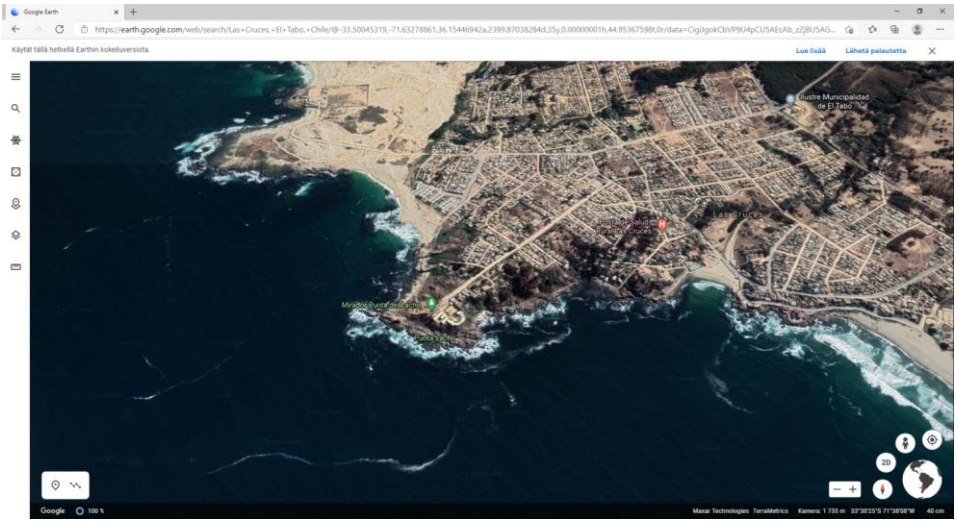

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***Morphological methods***

The *Gyrodactylus* specimens were removed from the ethanol preserved fish using a 10 µl pipette under a stereomicroscope. From the two fish species, 24 specimens of *Gyrodactylus* were found and analyzed in this study. The worms were cut into two parts. The opisthaptors were prepared for morphological examination and the rest of the body was kept in 96 % ethanol for molecular analysis.

The haptors were partially digested by proteinase K in a final concentration of 60 µg/ml prior to their preservation on slide in ammonium picrate-glycerin [5]. Measurements of the haptoral hard parts were taken with a microscope and digital camera (Nikon Coolpix 950, or Nikon Optiphot-2) and measured using the interactive measuring system IMT iSolution Lite (ver.7.4, IMT iSolution Inc.). The holotype of each species was drawn and the available specimens measured. The microscopy slides were deposited into the collection of the Finnish National History Museum (LUOMUS) in Helsinki University.

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***Molecular methods***

*DNA extraction, amplification and sequencing*

The DNA was isolated by digesting the parts of the single parasite specimens in 10 µl of a lysis buffer (1× PCR buffer, 0.45% (v/v) Tween 20, 0.45% (v/v) NP 40, and 60 µg/ml proteinase K). The tubes were incubated at 65 °C for 25 minutes to allow proteinase K digestion and then for 10 minutes at 95 °C to denature the proteinase before being cooled down to 4 °C. Aliquots of 2 µl of

this lysate were used as templates for PCR amplification. The remaining impurities in the lysate did not interfere with the PCR process.

The entire ITS region of the ribosomal DNA array (spanning ITS1-5.8S-ITS2 and flanking terminal fragments of the 18S and 28S rRNA genes) was amplified with primers ITS1F (5'-GTTTC CGTAG GTGAA CCT-3') [12] and ITS2R (5'-GGTAA TCACG CTTGAA TC-3') [13]. The PCR reaction contained 2 µl of lysate, 1×PCR buffer, 2 mM $MgCl_2$, 1 µM of each primer, 200 µM of each dNTP and 0.4 units of the *Taq* polymerase (Fermentas, Vilnius, Lithuania) in a final volume of 20 µl. The amplification mixtures were heated for 3 min at 95°C, subjected to 37 cycles (94°C, 48°C and 72°C for 1 min each), heated for 7 min at 72°C and cooled down to 4°C. The amplified fragments were purified from the agarose gel and sequenced directly with two additional primers, ITS1R (5'-ATTTG CGTTC GAGAG ACCG-3') and ITS2F (5'-TGGTG GATCA CTCGG CTCA-3') as described earlier [14].

The complete ITS1-5.8S rDNA-ITS2 sequence was produced from 24 parasites. The sequence alignments were made using the Clustal and Muscle programs implemented in MEGA7 [15]. Large parts of both the ITS segments were too diverse to be reliably aligned globally, and the phylogenetic conclusions (Fig. 2) are based on the collection of species selected on the basis of 5.8S sequence. The “First hairpin of ITS2” (Fig. 1) was predicted in http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi, University of Vienna. The phylogenetic trees were constructed for 5.8S + ITS2 sequences via the neighbor joining method based on Kimura's two parameter distance, as implemented in the MEGA7 program package. The alignment used for tree construction is given in Supplementary file B, in FASTA format. A Maximum Likelihood tree was also calculated for comparing the topology. The bootstrap values of both trees are given in Fig. 1.

A broader phylogenetic comparison of the new species was conducted among a collection of all the deposited GenBank entries of gyrodactylids (*Gyrodactylus*, *Paragyrodactylus*, *Macrogyrodactylus*, *Gyrdicotylus*, *Swingleus*). GenBank was consulted on June 12, 2020. The ITS1–5.8S–ITS2

sequence is not well suited for deeper phylogenies, because of accumulating problems and loss of graduality with increasing genetic distances [16]. We utilized only the higly conservative and systematically informative 5.8S RNA fragment (157 bp, [16].) and the structurally conservative beginning of the ITS2 including the first hairpin (total length 234 positions). The tree based on number of differences and neighbor joining is available in the Supplementary file A.

## Results

The six small specimens of clingfish, *Sicyases sanguineus*, examined were infected with *Gyrodactylus*. The monogeneans were found in the sucker of the fish, which is formed by the pectoral fins and maintains water when the fish is stuck on the rock in the surf zone [17, 18]. Thirteen specimens of *Gyrodactylus* were selected and analyzed. The ITS sequences were all identical, and the species are hereinafter described as *G. amphibius* sp. nov.

Further, we inspected the fins of a single specimen of combtooth blenny *S. viridis* caught from a tidal pond by a hand net. Eleven monogeneans were successfully sequenced and labelled as *Sca*. The sequencing allowed them to be classified into four groups. Specimen *Sca5* was *G. amphibius* sp. nov., specimen *Sca6* was unique and are hereinafter named as *G. zietarae* sp. nov. Five specimens (*Sca3, Sca 7, Sca 8, Sca 22,*and *Sca 23*) are described as *G. scartichthi* sp. nov., and four (*Sca9, Sca 10, Sca 14,* and *Sca 21*) are named as *G. viridae* sp. nov.

The outline characteristics of the ITS1–5.8S–ITS2 segment are in some aspects, comparable with, for instance, the data available in GenBank of the *wageneri* group of the subgenus *Gyrodactylus* (*Limnonephrotus*). The length variation in the ITS1 segment is very large and apparently created by deleted or inserted blocks, perhaps during recombinatory scrambling (Table 1). Due to the length variation, the alignment of the ITS1 is not possible, but it gives weight to the species identification.

**Table 1.** Variation in the length of the segments (bp) in the ITS region and GenBank accession numbers

| Species | ITS1 | 5.8S | ITS2 | GenBank | Reference |
|---|---|---|---|---|---|
| *G. amphibius* sp. nov. | 512 | 157 | 412 | MT675961 | Present study |
| *G. chileani* | 437 | 157 | 402 | JQ045347 | [19] |
| *G. orecchiae* | 513 | 157 | 405 | FJ013097 | [20] |
| *G. proterorhini* | 404 | 157 | 365 | MK584285 | [26] |
| *G. scartichthi* sp. nov. | 514 | 157 | 412 | MT675962 | Present study |
| *G. viridae* sp. nov. | 530 | 157 | 412 | MT675963 | Present study |
| *G. zietarae* sp. nov. | 486 | 157 | 412 | MT675964 | Present study |

The length of the 5.8S ribosomal RNA segment is the same, 157 bp, as in all the species in this genus (and widely among flatworms). The phylogenetic hypothesis constructed for the species sharing the identical 5.8S (Fig. 1) opens a very global view, connecting species from the Southern and the Northern Hemisphere, and from the Atlantic and Pacific Oceans.

The length variation of the ITS2 in the Chilean species is constrained, it is 402-412 bp, in spite of numerous 1–2 nucleotide indels and *blocks* of divergent sequence, in contrast to single-nucleotide substitutions (Supplementary file B).

The global phylogenetic position of *G. chileani* was determined by Mendoza-Palmero et al. [22]. In the Supplementary file A, a phylogenetic hypothesis based on 5.8S and short conservative (alignable) fragment of ITS2 is displayed. The five Chilean species and the three European relatives have a common root (71 % bootstrap support) in this hypothesis based on short sequence.

158 However, an inner circle comparison of species sharing *identical* 5.8S is more illuminating (Fig. 1).
159 In the tree based on the conservative 5.8S RNA and the ITS2 the *orecchiae* group has 100 %
160 support. The coding ribosomal 5.8S RNA is identical in the Chilean species *G. zietarae* sp. nov., *G.*
161 *chileani*, *G. scarthichthi* sp. nov, and *G. viridae* sp. nov., as well as in the Mediterranean *G.*
162 *orecchiae* [20] and the Ponto-Caspian *G. proterorhini* sampled in Belgium [25] and in the Black
163 Sea [26]. The divergent species *G. amphibius* sp. nov. differs from this by a single nucleotide
164 substitution, transversion G>T in site 112 (Supplementary file C, Fig. S2). This substitution is
165 shared only with *G. mediotorus*, which is not related (Supplementary file A), and thus has no
166 phylogenetic value.

167 The marine species described in Antarctica: *G. antarcticus*, *G. nudifronsi* and *G. coricepsi* from
168 Admiralty Bay and the Weddell Sea [12, 21] cluster together (100%) with species from the
169 Northern Hemisphere: *G. longipes*, *G. robustus*, *G. flesi*, G. *perlucidus* and *G. mariannae*. There is
170 aless condensed sister group is composed ofspecies from the Northern Hemisphere only, both from
171 Atlantic and Pacific: *G. alexanderi* (Holarctic), *G. branchicus*, *G. rarus*, *G. flesi* and *G. robustus*
172 (Northeast Europe and the Baltic Sea), *G. longipes*, *G. perlucidus*, *G. rugiensoides*, and *G.*
173 *rugiensis* (Northern Atlantic), and *G. medaka* (Japan).

174 Of interest with respect of the salinity thema is that *G. lotae* and *G. alexgusevi* also belong to this
175 5.8S cluster, but they are found in Eurasian freshwaters, together with their host *Lota lota*, a
176 Gadidae moved to inland. Similarly, *G. mariannae* is a European freshwater parasite on *Cottus*
177 *poecilopus*, which belongs in mostly marine family Cottidae.

178 In a close group of 5.8S diverged by a single parsimoniously informative nucleotide (32 U>C) are
179 marine relatives from the Northern Hemisphere — *G. corti*, *G. cyclopteri*, *G. adspersi*, *G. aideni*, *G.*
180 *pleuronecti*, *G. marinus* and *G. pterygialis* — and again, the freshwater parasite *G. hrabei* on *Cottus*

*poecilopus*. These species were included in the rough phylogenetic comparison in Supplementary file A.

A systematically highly informative secondary structure (stem-loop-stem) called the first hairpin of the ITS2 (UCGCGAC-GCUUAAUUA-GUCGCGG) is unique and shared with all the five Chilean species (Fig. 2). It separates the Chilean group from the large group of species that are identical or almost identical in 5.8S listed above, and it is unique among the sequences deposited in GenBank until October 2020.

The related species *G. proterorhini* and *G. sp* on *Gobius niger* differ from the five Chilean species by a compensatory pair of transitions (C>T[7] and G>A[17]: UCGCGAU-GCUUAAUUA-**AU**CGCGG). The canonical Watson-Crick pair C-G has changed by two transitions via intermediary (and tolerated) C—G > U—G > U—A, maintaining the hairpin structure (Meer et al. 2010).

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The species *G. orecchiae* is more different, with a compensatory pair of transversions (A>U[6] and U>A[18]). The Watson—Crick pair A—U can only change to U—A by two transversions, and the intermediary form disturbs the stem structure of the hairpin. Such combinations are never found, so they must have lowered fitness. The transition (C>U[21]) maintains the structure, but may not be optimal. All these three versions of the first hairpin in the *orecchiae* group are unique for this clade, among 110 species of *Gyrodactylus* and 56 other species of flatworms (data in GenBank).

**Description of the new species**

Gyrodactylidae Cobbold, 1864

Genus *Gyrodactylus* Nordmann, 1832

Species group suggestion: *orecchiae* group, anchoring the new species with the first molecular description of *Gyrodactylus orecchiae* Paladini, Cable, Fioravanti, Faria, Di Cave, Shinn 2009 on *Sparus auratus* from the Mediterranean Sea [23].

***Gyrodactylus amphibius* sp. nov.**

*Type-host*: clingfish *Sicyases sanguineus* Müller & Troschel, 1843

*Other or accidental host*: combtooth blenny *Scartichthys viridis* Valenciennes, 1836

*Type locality*: Las Cruces (33.48333°S; 71.616667° W), Central Chile.

*Site on host*: The ventral sucker formed by pectoral fins

*Type-material*: Type-specimens (holotype and three paratypes) slides of haptors are deposited in the Finnish National History Museum LUOMUS in Helsinki University, specimen numbers MZH118031 (*Sic14* holotype) and MZH118032, MZH118036, MZH118037 (paratypes).

*Representative DNA sequences*: GenBank accession number for ITS (based on nine specimens) MT675961.

*Etymology*: The name *Gyrodactylus amphibius* of the species is derived from the ecology of the host and parasite, describing (in Latin) its amphibious habit.

*Description* (Fig. 4)

[Based on 5 specimens: four from the type-host and one from the *Scartichthys viridis*]. Total length of anchor is not comparable genus-wide because the root is folded. Anchor with fold, length between extreme points 36.7–40.9 μm; anchor root long (10.2–11.9 μm) and bent ventrally, anchor shaft pronounced (24.5–34 μm). Ventral bar (23-31 μm) is with big prominent rounded processes looking as "mouse ears" and medium size triangular membrane (12-13 μm). Dorsal bar is missing.

Marginal hook with handle is long (20-27 µm), marginal hook sickle 4.98-5.49 µm long with rhomboid toe and heel; bent backwards; filament loop long, extends almost to middle of handle; transition of shaft to point not distinct.

*DNA characteristics*: Total length of the ITS1-5.8S-ITS2 segment amplified, sequenced and aligned (from CAAATT to CTAAGT) was 1072 nucleotides. The 157 bp long 5.8S ribosomal RNA sequence of *Gyrodactylus amphibius* sp. nov. differed from the other new species presented here by a single transversion G > T in site 112 and is identical with *G. mediotorus* King, Marcogliese, Forest, McLaughlin and Bentzen, 2013 (KF178301). This 5.8S version was unique in the GenBank collection of *Gyrodactylus* sequences until May 2019. However, *G. mediotorus* is not a close relative of the species group treated in this paper, as concluded from the ITS2. We conclude that this G > T is a genuine paraphyletic mutation, which has occurred twice in the *Gyrodactylus* material in GenBank.

*Remarks:* The dorsal bar is missing, the heel and toes in marginal hooks prominent. The shapes of the hamuli resemble those of *G. chileani* and *G. orecchiae* [19, 23]. The hamulus roots folded and the points extend almost to the half-length of the shafts. The ventral bar has much larger processes than in *G. chileani* and almost the same as in *G. orecchiae*. The marginal hooks with the toe rhomboid shape resemble to *G. orecchiae.* Both the marginal hook total and sickle are much longer than those of *G. chileani* and *G. orecchiae*. *Gyrodactylus amphibius* sp. nov. is similar to *G. proterorhini* [24, 25, 26] with the sclerotized extra plates. The morphology of *Gyrodactylus amphibius* sp. nov. is very distinct from that of *G. mediotorus.*

*Ecological note:* The host of *G. amphibius* sp. nov. is the Chilean clingfish that inhabits the rocky coasts of Chile and Southern Peru [9]. The fish survives above the high tide in the surf zone, attached to rocks by the sucker for a long time, and breathes air through the skin [18]. While the sucker maintains moisture, the osmotic conditions may become challenging for the tiny worm.

***Gyrodactylus scartichthi* sp. nov.**

*Type-host*: *Scartichthys viridis*

*Other host*: Not known.

*Type locality*: Las Cruces (33.48333°S; 71.616667° W), Central Chile.

*Site on host*: Fins of one fish were inspected

*Type-material*: Type-specimen (holotype): slide of haptors is deposited in the Finnish National History Museum in Helsinki University (LUOMUS), specimen number MZH118056

*Representative DNA sequences*: GenBank accession number for ITS (based on three specimens) MT675962.

*Etymology*: The species name is based on the host genus.

*Description* (Fig. 4) is based on two specimens from the type host *Scartichthys viridis*. Haptor ovate. Anchor long (47.2 -49.5 µm) with fold; anchor root long (8.28-8.69 µm) tends to bend ventrally, anchor shaft pronounced (24.9-26.18 µm). Ventral bar (15.82-19.52 µm) with long, round processes and medium triangular membrane (9.86-12.64 µm). Dorsal bar delicate, with 20.79-21.95 µm length and 2.38-2.8 µm wide. Marginal hook 19.21-20.16 µm with small hook sickle (3.84-3.98 µm); marginal hook shaft leaning; marginal hook point extending toe and pointing backwards; filament loop long, extends almost to middle of handle; transition of shaft to point not distinct.

*DNA characteristics*: Total length of the ITS1-5.8S-ITS2 segment amplified, sequenced and aligned (from CACATG to TGAAGT) was 1074 nucleotides.

*Remarks: G. scartichthi* sp. nov. differs from other newly revealed *Gyrodactylus* species given a very big and round “mouse ears” in the ventral bar. They are also much larger than in both *G.*

*chileani* and *G. orecchiae*. The shape of the hamuli as in *G. amphibius* sp. nov., resemble those of *G. chileani* and *G. orecchiae* in the hamulus roots folded and the presence of dorsal bar. The length and width of the dorsal bar are similar to those in *G. chileani* and *G. orecchiae* [19, 23].

*Ecological note:* The species was found together with the other three species described here, on a single individual host.

***Gyrodactylus viridae* sp. nov**.

*Type-host*: *Scartichthys viridis*

*Other host*: Not known.

*Type locality*: Las Cruces (33.48333°S; 71.616667° W), Central Chile.

*Site on host*: fins

*Type-material*: Holotype: slide of haptor is deposited in the Finnish National History Museum in Helsinki University (LUOMUS), specimen numbers MZH118062 (*Sca21* holotype).

*Representative DNA sequences*: GenBank accession number for ITS is MT675963.

*Etymology*: Named according to the species name of the host

*Description* (Fig. 4). Based on one specimen. Haptor ovate. Anchor long (53.23 µm) with fold; anchor root long (14.89 µm) and bent ventrally, anchor shaft pronounced (32.28 µm). Ventral bar (26.76 µm) with long, round processes and medium triangular membrane (13.51 µm). Dorsal bar delicate (23.25 µm) and wide (1.6 µm). Marginal hook long (26.59 µm) with delicate marginal hook sickle (5.46 µm); marginal hook shaft leaning; marginal hook point extending toe and pointing backwards; filament loop long extends almost to middle of handle; transition of shaft to point not distinct.

*DNA characteristics*: Total length of the ITS1-5.8S-ITS2 segment amplified, sequenced and aligned (from CACATT to TGAAGT) was 1090 nucleotides. It was obtained from four specimens *Sca9, Sca 10, Sca 14*, and *Sca 21*. Systematically informative secondary structure, the first hairpin in the ITS2 (TCGCGAC-GCTTAATTA-GTCGCGG) is shared with all five Chilean species and separates them from all other species available in GenBank until May 2019.

*Remarks*: the hamuli roots are the largest among the investigated group. They are observed to turn inward over the ventral bar processes. The ventral bar of *G. viridae* also has big round "mouse ears". The dorsal bar is missing, as in *G. amphibius.*

*Ecological note:* The species was found together with the other three species described here, on the single host individual.

***Gyrodactylus zietarae* sp. nov.**

*Type-host*: *Scartichthys viridis*

*Other host*: Not known.

*Type locality*: Las Cruces (33.48333°S; 71.616667° W), Central Chile.

*Site on host*: fins

*Type-material*: Type-specimen (holotype) slide of haptor is deposited in the Finnish National History Museum in Helsinki University (LUOMUS) with number MZH118050 (*Sca6*).

*DNA sequence*: GenBank accession number for ITS is MT675964.

*Etymology*: The name is in honor to Professor Marek Ziętara, Gdansk, Poland, who is one of the pioneers of the molecular taxonomy and systematics of *Gyrodactylus*.

*Description* (Fig. 4) Based on one specimen *Sca6*. Haptor ovate. Anchor long (45 µm) with fold; anchor root long (19.7 µm) and bent ventrally, anchor shaft pronounced (25.5 µm). Ventral bar (19.2 µm) with long, round processes and medium triangular membrane (10.3 µm). Dorsal bar delicate (21.3 µm) and wide (1.9 µm). Marginal hook is 20.67 µm length with delicate marginal hook sickle (4.3 µm); marginal hook shaft leaning; marginal hook point extending toe and pointing backwards; filament loop long, extends almost to middle of handle; transition of shaft to point not distinct.

*DNA characteristics*: Total length of the ITS1-5.8S-ITS2 segment amplified, sequenced and aligned (from CACATT to AAAAGT) was 1046 nucleotides.

*Remarks:* We describe the species based on a single individual, relying on the standard DNA marker which shows that the specimen is unique among all the molecularly defined *Gyrodactylus* species, and also different enough from their relatives, which are described here. VThe ventral bar has big round “mouse ear” extensions. The dorsal bar is quite thick. *G. zietarae* sp. nov. is similar to *G. amphibius sp. nov.* and *G. proterorhini* [24, 25] in terms of the presence of the sclerotized extra plates.

*Ecological note: G. zietarae* sp. nov. was found together with the other three species described here, on the fins of the single host individual.

**Discussion**

Malmberg’s [5] subgenera and systematics of *Gyrodactylus* were based on osmoregulatory organs. Therefore, it may be interesting to search, describe, and compare the extremes of salinity tolerance of these parasites. Two subgenera, *G.* (*Limnonephrotus*) and *G.* (*Gyrodactylus*) were restricted to freshwater. With a global scope, the latter probably should be divided to sister groups, living in Palaearctic, Asian and African continents [32] (Reyda et al., 2019)

Other subgenera are mostly marine. Paladini et al. [20] described a hypersalinity tolerant *G. salinae* Paladini, Shinn & Huyse, 2011 from the Mediterranean banded killifish *Aphanius fasciatus* Valenciennes 1821 (Cyprinodontinae) collected in salt extraction pools, in Northern Italy. In the molecular phylogenetic framework by ITS, the species was close to the eel parasite *G. anguillae* placed in the subgenus *Gyrodactylus* (*Neonephrotus*) by Malmberg [5]. The ITS of *G. anguillae* Ergens, 1960 [27] is close to several marine parasites of the *rugiensis* group on the North Sea and Mediterranean gobies, which were tentatively placed into *G.* (*Paranephrotus*) [28, 29]. Together, the species of the *rugiensis* group, including *G. salinae* and *G. anguillae* form a rather consistent group (Supplementary file A), which is a sister group of the freshwater subgenus *G.* (*Limnonephrotus*). *G. salinae* has an uniquet segment 124-129 in the 5.8S, but the first hairpin fits with the *rugiensis* group and with *G. leptorhynchi*, *G. eyipayipi*, *G. corleonis* and *G. neretum*.

The monogenean *G. magadiensis* dos Santos, Maina & Avenant-Oldewage 2019 on a Magadi tilapia *Alcolapia grahami* Boulenger ,1912 (Cichlidae) from the alkaline soda lake Lake Magadi in Kenya may represent one extreme in the osmotic stress challenge, as well as pH problems, high temperature and low oxygen [30]. Using BLAST, it was considered closest to *G. branchicus* Malmberg, 1964 [14] on the Scandinavian three-spined stickleback *Gasterosteus aculeatus* Linnaeus, 1758 [14]. However, based on a wider comparison, *G. magadiensis* is a novel branch in the genus, carrying a unique 5.8S and the first hairpin. The nearest relatives are *G. lamothei* and *G. katamba* from Mesa Central, Mexico [Rubio-Godoy et al. 2016] (Supplementary file A). There are also observations of *Gyrodactylus* on amphibians (reviews in [31, 32]), but these are restricted to aquatic larval stages in freshwater. The few *Gyrodactylus* parasites on amphibians are not really amphibious.

The five Chilean parasite species from the same intertidal to supratidal habitat are phylogenetically connected with some species from the Ponto-Caspian and North Sea (Atlantic) regions, as was

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already observed by Ziętara *et al.* [19]. This may be an unexpected connection, but it gives meaning to the apparently sporadic taxonomic descriptions when supported by DNA barcoding. Objective and testable connectivity is the power of molecular systematics, even if the ITS is far from optimal for constructing deep phylogenies. It demonstrates close relatedness and separates sexually isolated taxons, but fails to reliably estimate wider distances. For species delimitation and identification, the ITS segment is optimal, because the available primers are more conservative than the primers utilized for rapidly developing mitochondrial markers.

The parasite *G. proterorhini*, originating in the Black Sea, is a freshwater colonizer in Europe, and has been recorded not only on the major invasive host, the Western tubenose goby *Proterorhinus semilunaris* [25] but also on many host species, all family Gobidae, such as racer goby *Babka gymnotrachelus*, the monkey goby *Neogobius fluviatilis*, the round goby *N. melanostomus* and the bighead goby *Ponticola kessleri* [33, 34]. These observations should be confirmed via molecular analysis.

As the first *Gyrodactylus* species reported from Chile, *Gyrodactylus chileani* Ziętara, Lebedeva, Muñoz & Lumme 2012 was described on *Helcogrammoides chilensis* Cancino 1960 (Tripterygidae) [19]. The four *Gyrodactylus* species we had found from the same environment proved to be quite closely related phylogenetically. These four new *Gyrodactylus* species are the only known gyrodactylid parasites of the endemic fishes *S. sanguineus* (Gobiesocidae) and *S. viridis* (Blennidae). The *S. sanguineus* hosted only one gyrodactylid species on the ventral sucker while the *S. viridis* had the all four parasite species on the fins (dorsal and pectoral). The Chilean guild of *Gyrodactylus* parasites is connected by the same habitat rather than by the systematic relatedness of the host fish.

The novel parasite species have some unusual morphological features. *G. amphibius* sp. nov. and *G. zietarae* sp. nov. have extra sclerotized plates situated anterior to the bent roots of the hamuli. Such extra plates were also found in *G. proterorhini* [25], parasitizing on different Gobiidae through the

383 basins of the Black and Caspian Seas. The ventral bars of *G. amphibius* sp. nov. and *G. viridae* sp.
384 nov. were missing, a characteristic found in the *unipons* group of species in the Northern
385 Hemisphere [5]. To our knowledge, no named members of *unipons* group have been sequenced for
386 ITS.
387 Worth noting but not surprising is that most of the parasite species found in intertidal fish from
388 Chile have been new species. For example, in the blenny *S. viridis*, several parasite species of
389 different taxonomic groups has been described since 2009: a nematode (*Pseudodelphis chilensis*
390 Muñoz, 2010), a copepod (*Colobomatus tenuis* Castro & Muñoz, 2009), two monogeneans
391 (*Microcotyle sprostonae* and *M. chilensis* Muñoz & George-Nascimento, 2009 and two digeneans
392 (*Monorchimacradena viridis* Muñoz, George-Nascimento & Bray, 2017 and *Megasolena littoralis*
393 Muñoz, George-Nascimento & Bray, 2017). Several parasites are in a few host species of the same
394 habitats [35–38]. Moreover, a study of parasite communities of a fish assemblage from the intertidal
395 rocky zone of Central Chile [39] indicated that the endemism (fish and parasites) is high in this
396 habitat. Also, the resident intertidal fish were characterized by their own parasite species, meaning
397 that their transmissions might be restricted to the intertidal zone.
398 Consequently, we expect that further investigation and the power of molecular systematics applied
399 to parasites of intertidal fish will extend the knowledge about the parasite species diversity. Also,
400 the biogeographic connections of the South-Eastern Pacific with other regions of the world may be
401 revealed by the taxonomic relationships of the parasites. The phylogenetic jump from the Black Sea
402 to the Chilean coast, or from the Barents Sea to Antarctica is already quite surprising [40].

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406 **Compliance with Ethical Standards**

407 **Conflict of interest**

The authors declare that they have no competing interests.

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**Legends to figures:**

**Fig. 1.** Phylogenetic hypothesis based on the whole 5.8S–ITS2 segment (the 560 bp sequence alignment in Supplementary Material B). All the species except *G. amphibius* sp. nov. in the tree have identical 5.8S RNA genes. Neighbor joining tree with pairwise deletions and K2P distance estimate and bootstrap values from 500 repeats. The bootstrap values from Maximum Likelihood tree (in italics) are added.

**Fig. 2.** The structure of the first hairpin in ITS2 among the species having *identical* 5.8S sequence. The nucleotide changes in comparison to the *G. perlucidus- G. longipes* cluster are marked as bold. The phylogenetic hypothesis of these species is displayed in Fig. 3.

**Fig. 3.** Opisthaptoral central hook complex and marginal hooks of the four new salinity tolerant species of *Gyrodactylus* from the Chilean coast (scale bars 10 µm.

**Supplementary material**

**Supplementary file A.** The phylogenetic hypothesis (.jpg file) of global collection of 5.8S + beginning of ITS2 (234 bp) to suggest the phylogenetic position of *G. magadiensis*, *G. salinae* and

555 *G. amphibius* adapted to high salinity. The nearest relatives of the Kenyan *G. magadiensis* are *G.*
556 *lamothei* and *G. katamba* from Mesa Central, Mexico (Rubio-Godoy et al., 2016).
557
558 **Supplementary file B.** The FASTA alignment 5.8S + ITS2 sequences of the *Gyrodactylus* species
559 sharing identical 5.8S ribosomal DNA used for phylogenetic reconstruction in Fig. 2.
560
561 **Supplementary file C.** Supplementary figures SFig1, SFig2, SFig3 in .pdf format