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ORIGINAL PAPER

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 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 from two fish species, the clingfish *Sicyases sanguineus* Müller & Troschel, 1843 and the combtooth blenny *Scartichthys viridis* Valenciennes, 1836. The morphology was described by 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 with the previously described *G. chileani* Zietara *et al.* 2012 on 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 genus *Gyrodactylus* von Nordmann, 1832, is rather conservative, but the spectrum of ecophysiological adaptations is wide, as is the "invisible" DNA variation [42]. 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., [4, 9, 15]). Malmberg [18] 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 [7], studied, for example, among free-living nematodes [8]. Fish parasites cannot make the salinity orientation independently, but have to rely on their host [19].

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In this paper, we describe four new species of *Gyrodactylus* living in osmotically challenging environment on the Chilean rocky coast, utilizing the resolution of the sequencing 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 common root, or whether the tolerance evolved independently in separate branches of the phylogenetic tree.

We describe the first genuinely amphibious *Gyrodactylus* on the amphibious clingfish *Sicyases sanguineus* Müller & Troschel 1843 from the Chilean coast [1]. Evolution of amphibious behavior has occurred repeatedly in ecologically diverse fish families [30]. 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 [30].

Materials and Methods

The collection occurred in Las Cruces, Central Chile (33° 29.230 S–71° 38.573 W) on April 2012 [28]. Six small (< 3 cm) juvenile specimens of the clingfish *Sicyases sanguineus* Müller & Troschel 1843 (local name pejesapo) were caught on the tidal rocks one by one using a hand net. Also, one specimen of a combtooth blenny, *Scartichthys viridis* Valenciennes 1836 (local name borrachilla), was caught from tidal ponds. The fins were cut from each fish and stored in ethanol in a separate tube.

Morphological Methods

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

The body parts with haptors were partially digested by Proteinase K in a final concentration of 60 g/ml prior to their preservation in ammonium picrate-glycerin [18]. Measurements of the haptoral hard parts were taken with a microscope and digital camera (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 were measured. The microscopy slides were deposited into the collection of the Finnish National History Museum LUOMUS in Helsinki University.

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 min to allow proteinase K digestion and then for 10 min 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′) [36] and ITS2R (5′-GGTAA TCACG CTTGAA TC-3′) [39]. 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 [43].

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 [16]. Large parts of both the ITS segments were too diverse to be reliably aligned globally, and the phylogenetic conclusions (Fig. 1) are based on the collection of species selected on the basis of 5.8S sequence. The "First hairpin of ITS2" (Fig. 2) was predicted using ViennaRNA Package, V. 2.4.17 (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi, University of Vienna). The phylogenetic trees were constructed for 5.8S + ITS2 sequences by the Neighbor Joining method based on Kimura's two parameter distance, as implemented in the MEGA7 program package. The FASTA alignment used for tree construction is given in Supplementary file A. 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*).

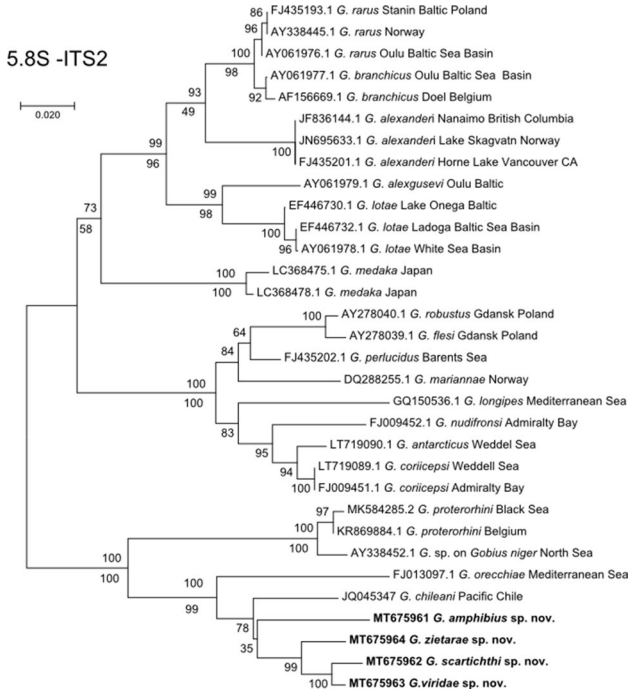

Fig. 1 Phylogenetic hypothesis based on the whole 5.8S–ITS2 segment (the 560 bp sequence alignment in Supplementary Material B). All the species except *G. amphibius* sp. nov. in the tree have identical 5.8S RNA genes. Neighbor joining tree with pairwise deletions and K2P distance estimate and bootstrap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

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 [40]. We utilized only the highly conservative and systematically informative 5.8S RNA fragment (157 bp, [40].) 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 B.

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 [6, 20]. Thirteen specimens of *Gyrodactylus* were picked off 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 *Scartichthys viridis* caught from a tidal pond by hand net. Eleven monogeneans were found, labeled as *Sca* and successfully sequenced. The sequencing allowed them to be classified into four groups. Specimen *Sca5* was *G. amphibius* sp. nov., specimen *Sca6* was unique and is hereinafter named as *G. zietarae* sp. nov. Five specimens (*Sca3, 7, 8, 22, 23*) are described as *G. scartichthi* sp. nov., and four (*Sca9, 10, 14, 21*) are named as *G. viridae* sp. nov.

The outline characteristic of the ITS1-5.8S-TS2 segment is in some aspects comparable with, for instance, the data available in GenBank of *wageneri* group of 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

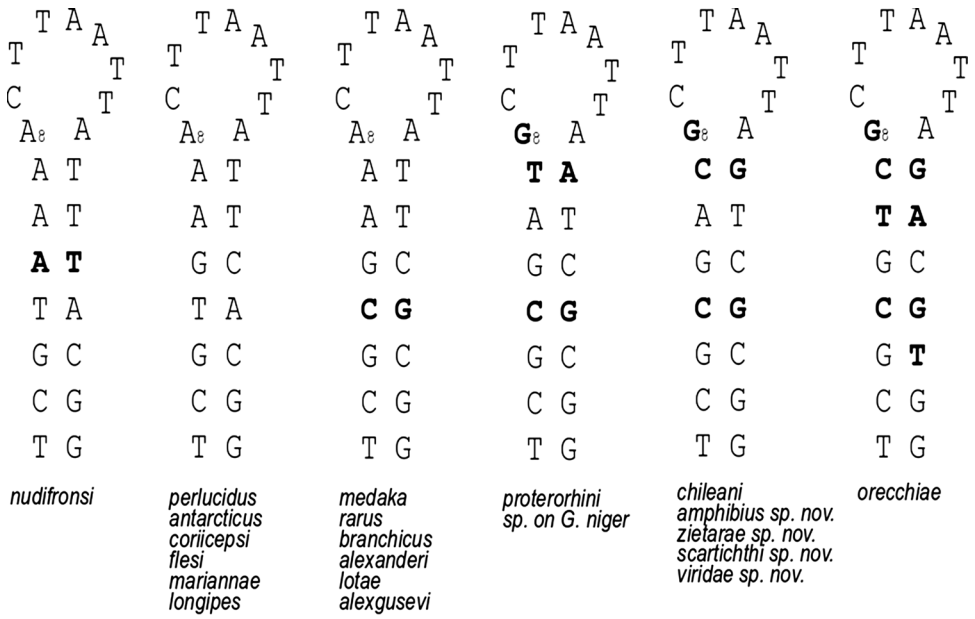

Fig. 2 The structure of the first hairpin in ITS2 among the species having identical 5.8S sequence. The nucleotide changes in comparison to the *G. perlucidus–G. longipes* cluster are marked as bold. The phylogenetic hypothesis of these species is displayed in Fig. 3

(Table 1). Due to the length variation, the alignment of the ITS1 is not possible, but it gives weight to the species identification.

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 A).

The global phylogenetic position of *G. chileani* was determined by Mendoza-Palmero *et al.* [22]. In the Supplementary file B, 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.

However, an inner circle comparison of species sharing identical 5.8S is more illuminating (Fig. 1). In the tree based on the conservative 5.8S RNA and the ITS2, the *orecchiae* group has 100% support. The coding ribosomal 5.8S RNA is identical in the Chilean species *G. zietarae* sp. nov., *G. chileani*, *G. scarthichthi* sp. nov, and *G. viridae* sp. nov., as well as in the Mediterranean *G. orecchiae* [20] and the Ponto-Caspian *G. proterorhini* sampled in Belgium [13] and in the Black Sea [17]. The divergent species *G. amphibius* sp. nov. differs from this by a single-nucleotide substitution, transversion G > T in site 112 (Supplementary file C, Fig. S2). This substitution is shared only with *G. mediotorus*, which is not related (Supplementary file B), and thus has no phylogenetic value.

The marine species described in Antarctica: *G. antarcticus*, *G. nudifronsi* and *G. coricepsi* from Admiralty Bay and the Weddell Sea [11, 36] cluster together (100%) with species from the Northern Hemisphere: *G. longipes*, *G. robustus*, *G. flesi*, *G. perlucidus* and *G. mariannae*. There is a less condensed sister group composed of species from the Northern Hemisphere only, both from Atlantic and Pacific: *G. alexanderi* (Holarctic), *G. branchicus, G. rarus, G. flesi* and *G. robustus* (Northeast Europe and the Baltic Sea), *G. longipes, G. perlucidus, G. rugiensoides,* and *G. rugiensis* (Northern Atlantic), and *G. medaka* (Japan).

Of interest with respect to the salinity thema is that *G. lotae* and *G. alexgusevi* also belong to this 5.8S cluster, but they are found in Eurasian freshwaters, together with their host *Lota lota*, a Gadidae moved to inland. Similarly, *G. mariannae* is a European freshwater parasite on *Cottus poecilopus*, which belongs in mostly marine family Cottidae [38].

In a close group of 5.8S diverged by a single parsimoniously informative nucleotide (32 U > C) are marine relatives from the Northern Hemisphere—*G. corti, G. cyclopteri*, *G. adspersi*, *G. aideni*, *G. 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 B.

Systematically highly informative secondary structure (stem–loop–stem) called the first hairpin of the ITS2 (UCG CGAC-GCUUAAUUA-GUCGCGG) is unique and shared with all five Chilean species (Fig. 2, Supplementary file C, Fig. S2). 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 *Gyrodactylus* sp. on *Gobius niger* differ from the five Chilean species by a compensatory pair of transitions ($C > T^{7}$ and $G > A^{17}$: UCG CGAU-GCUUAAUUA-AUCGCGG). 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 [21].

The species *G. orecchiae* is more different, with 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 newer 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

Table 1 GenBank accession numbers and essential dimensions of the ITS (bp) of the *Gyrodactylus* species discussed here

Species	ITS1 length	5.8S	ITS2 length	GenBank	References
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

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 [32].

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° 29.230 S–71° 38.573 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 (Figs. 3 and 4) [Based on five 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: The total length of the ITS1-5.8S-ITS2 segment amplified, sequenced and aligned (from CAA ATT TO CTAAGT) was 1096 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

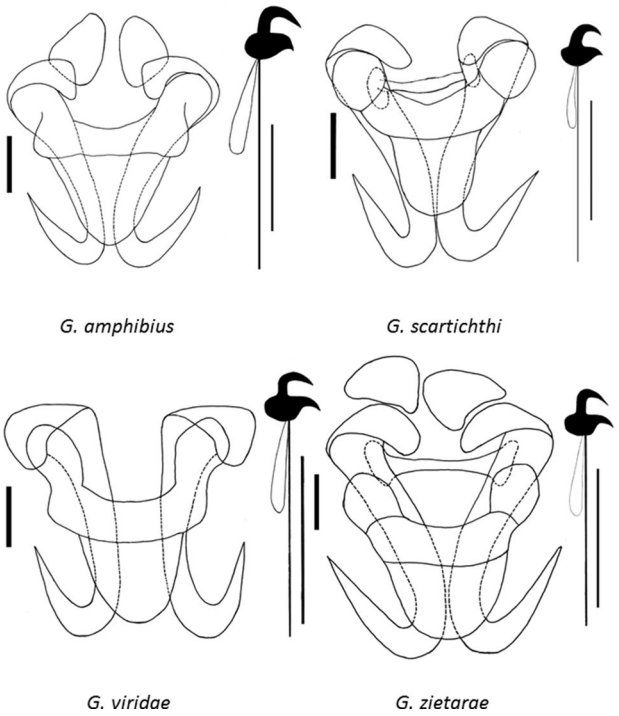

Fig. 3 Opisthaptoral central hook complex and marginal hooks of the four new salinity tolerant species of *Gyrodactylus* from the Chilean coast (scale bars 10 µm)

until June, 26 2020. 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, heel and toes in marginal hooks prominent. Shapes of hamuli resemble those of *G. chileani* and *G. orecchiae* [32, 41]. Hamulus roots folded and points extend almost to half-length of shafts. Ventral bar has much larger processes than in *G. chileani* and almost the same as in *G. orecchiae*. Marginal hooks with toe rhomboid shape close to *G. orecchiae.* Both marginal hook total and sickle are much longer than those of *G. chileani* and *G. orecchiae* (Table 2). *Gyrodactylus amphibius* sp. nov. is similar to *G. proterorhini* [13, 17, 34] with to regards 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., Chilean clingfish inhabits the rocky coasts of Chile and southern Peru [1]. The fish survives above the high tide in the surf zone, attached on rock by the sucker. The fish may dry occasionally, and breathes air [20]. While the sucker maintains moisture, the osmotic conditions may become challenging for the tiny worm.

Gyrodactylus scartichthi sp. nov.

Type-host: *Scartichthys viridis*

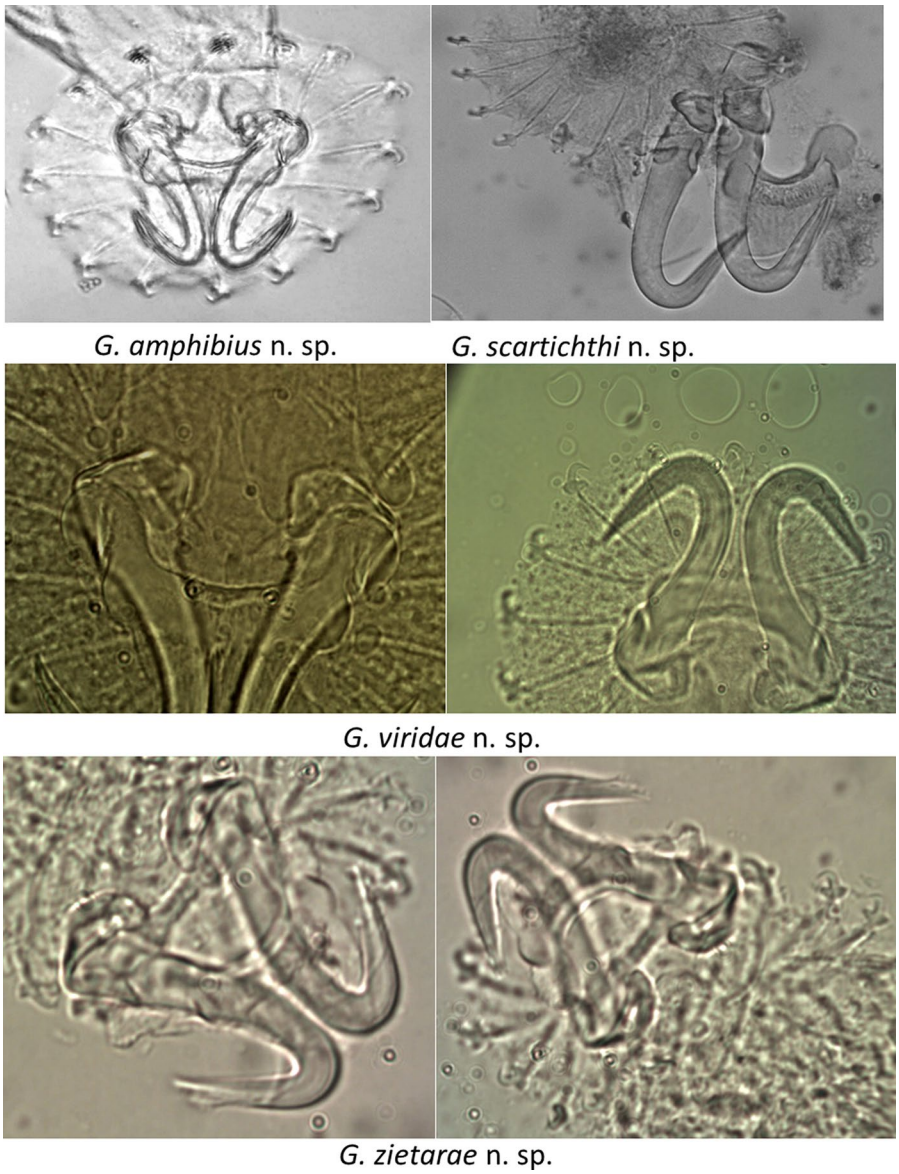

Fig. 4 Photo of the four new salinity tolerant species of *Gyrodactylus* from the Chilean coast

Table 2 Measurements (in µm) of some haptoral hard parts of *Gyrodactylus* spp. from *orecchiae* group

Species	*G. amphibius* sp. Nov	*G. scartichthi* sp. nov	*G. viridae* sp. nov	*G. zietarae* sp. nov	*G. orecchiae*	*G. chileani*
References	Present study	Present study	Present study	Present study	[32]	[41]
Hamuli shaft length	24.5–34	24.9–26.2	32.28	25.5	20.2–22.6	23–27
Hamuli point length	17–20	17.6–18.5	22	17.7	14.6–16.2	17–20
Ventral bar length	23–31	15.8–19.5	26.76	19.2	18.6–22.0	16–20
Ventral membrane length	12–13	9.9–12.6	13.51	10.3	9.3–11.8	9–12
Dorsal bar length	–	20.8–22	–	21.3	15.5–18.2	12–17
Dorsal bar width	–	2.4–2.8	–	1.9	1.8–2.3	2–3
Marginal hook length	20–27	19.2–20.2	26.59	20.67	17.5–18.7	21–23
Sickle length	5 -5.5	3.8–4	5.46	4.3	3.0–4.0	4–5

Other host: Not known.

Type locality: Las Cruces (33° 29.230 S–71° 38.573 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 (Figs. 3 and 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 1098 nucleotides.

Remarks: G. scartichthi. sp. nov. differs from other newly revealed *Gyrodactylus* species by a very big round processes as "mouse ears" in the ventral bar. The ventral bar processes are also the much larger than in both *G. chileani* and *G. orecchiae*. The length and width of dorsal bar are larger than in *G. chileani* and *G. orecchiae* (Table 2).

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° 29.230 S–71° 38.573 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 (Figs. 3 and 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). 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 1114 nucleotides. It was obtained from four specimens *Sca9, 10, 14, 21*. Systematically informative secondary structure, the first hairpin in the ITS2 (TCG CGAC-GCTTAATTA-GTCGCGG) is shared with all five Chilean species and separates them from all other species available in GenBank until June, 26 2020.

Remarks: The hamuli roots are the largest among the investigated group (Table 2). They are observed to turn inwards over the ventral bar processes. *G. viridae* also has big processes of ventral bar as 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° 29.230 S–71° 38.573 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 of Professor Marek Ziętara, Gdansk, Poland, who is one of the pioneers of the molecular taxonomy and systematics of *Gyrodactylus*.

Description (Figs. 3 and 4) Based on one specimen. 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 1070 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 molecularly defined *Gyrodactylus* species, and also different enough from the relatives, which are described here. The ventral bar has big round "mouse ear" extensions. The dorsal bar is quite thick. Dorsal bar length exceeds that of other *orecchiae* group representatives (Table 2).*G. zietarae* sp. nov. is similar to *G. amphibius* sp. nov. and *G. proterorhini* [13, 17] with presence of the sclerotized extra plates.

Ecological note: G. zietarae sp. nov. was found together with the other three species described here, on 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 into sister groups, living in Palaearctic, Asian and African continents [35].

Other subgenera are mostly marine. Paladini *et al.* [33] 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 [18]. The ITS of *G. anguillae* Ergens, 1960 [10] is close to several marine parasites of the *rugiensis* group on the North Sea and Mediterranean gobies, which were tentatively placed into *G.* (*Paranephrotus*) [12, 14]. Together, the species of the *rugiensis* group, including *G. salinae* and *G. anguillae* form a rather consistent group (Supplementary file B), which is a sister group of the freshwater subgenus *G.* (*Limnonephrotus*). *G. salinae* has a unique 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 not only one extreme in the osmotic stress challenge, but also pH problems, high temperature and low oxygen [5]. Using BLAST, it was considered closest to *G. branchicus* Malmberg, 1964 [43] on Scandinavian Three-spined stickleback *Gasterosteus aculeatus* Linnaeus, 1758 [43]. However, on the basis of wider comparison, *G. magadiensis* is a novel branch in the genus, carrying unique 5.8S and the First hairpin.

The nearest relatives are *G. lamothei* and *G. katamba* from Mesa Central, Mexico [37] (Supplementary file B). There are also observations of *Gyrodactylus* on amphibians (reviews in [31, 35]), 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 Ponto-Caspian and North Sea (Atlantic) regions, as was observed already by Ziętara *et al.* [41]. 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 ITSis far from optimal for constructing deep phylogenies. It demonstrates close relatedness and separates sexually isolated taxons, but fails to reliably estimate wider distances. For a 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.

This suggests further research that the parasite *G. proterorhini* originating in the Black Sea is a freshwater colonizer in Europe, and recorded not only on the major invasive fish Western tubenose goby *Proterorhinus semilunaris* [13] but also on many host species, all Family Gobidae, like racer goby *Babka gymnotrachelus*, monkey goby *Neogobius fluviatilis*, round goby *N. melanostomus* and bighead goby *Ponticola kessleri* [23, 29]. 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) [41]. The four *Gyrodactylus* species we had found from the same environment proved to be phylogenetically quite closely related. These four new *Gyrodactylus* species are the only known gyrodactylid parasites of the endemic fishes *Sicyases sanguineus* (Gobiesocidae) and *Scartichthys viridis* (Blennidae). The *S. sanguineus* hosted only one gyrodactylid species on ventral sucker while the *S. viridis* had the all four parasite species on fins (dorsal and pectoral). The Chilean guild of *Gyrodactylus* parasites is connected by the same habitat rather than by systematic relatedness of the host fish.

The novel parasite species have some unusual morphological features. In *G. amphibius* sp. nov. and *G. zietarae* sp. nov., there are extra sclerotized plates situating anteriorly of the bent roots of the hamuli. Such extra plates were also found in *G. proterorhini* [13, 17] parasitizing on different Gobiidae through the basins of the Black and Caspian Seas. The ventral bars of *G. amphibius* sp. nov. and *G. viridae* sp. nov. were missing, a characteristic previously found in *unipons* group of species in the Northern

Hemisphere [18]. To our knowledge, no named members of *unipons* group have been sequenced for ITS.

Worth noting but not surprising is that most of the parasite species found in intertidal fish from Chile have been new species. For example, in the blenny *S. viridis*, several parasite species of different taxonomic groups have been described since 2009: a nematode (*Pseudodelphis chilensis* Muñoz, 2010), a copepod (*Colobomatus tenuis* Castro & Muñoz, 2009), two monogeneans (*Microcotyle sprostonae* and *M. chilensis* Muñoz & George-Nascimento, 2009) and two digeneans (*Monorchimacradena viridis* Muñoz, George-Nascimento & Bray, 2017 and *Megasolena littoralis* Muñoz, George-Nascimento & Bray, 2017). Several parasites are in a few host species of the same habitats [2, 24, 26, 27]. Moreover, a study of parasite communities of a fish assemblage from the intertidal rocky zone of Central Chile [25] indicated that the endemism (fish and parasites) is high in this habitat. Also, the resident intertidal fish were characterized by their own parasite species, meaning that their transmissions might be restricted to the intertidal zone.

Consequently, we expect that further investigation and the power of molecular systematics applied to parasites of intertidal fish will extend the knowledge about the parasite species diversity. Also, the biogeographic connections of the South-Eastern Pacific with other regions of the world may be revealed by the taxonomic relationships of the parasites. The phylogenetic jump from the Black Sea to the Chilean coast, or from the Barents Sea to Antarctica, is already quite surprising [3].

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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