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Molecular and clinical evidence of *Aeromonas hydrophila* and *Fusarium solani* co-infection in narrow-clawed crayfish *Astacus leptodactylus*

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ABSTRACT: Co-infections occur when a host is infected by 2 or more different pathogen types, either by secondary or simultaneous infections, and are very frequent in nature. In this study, 10 narrow-clawed crayfish *Astacus leptodactylus* with signs of disease were collected from Haft Baram Lake (Fars province, southern Iran). Samples of fluid from inside the intact abscess and melanized lesions in the cuticle were cultured aseptically onto brain heart infusion agar and Sabouraud dextrose agar for bacterial and fungal agents, respectively. After primary colony isolation for bacterial and fungal agents, the isolates were confirmed as *Aeromonas hydrophila* and *Fusarium solani*, using specific PCR methods based on 16S rDNA and internal transcribed spacer (ITS) rDNA sequences that produced a single band of 685 bp and 600 bp, respectively. Partial sequence analysis of the *F. solani* ITS showed 100% sequence identity among all our samples, as well as a close genetic relationship between this isolate (GenBank accession number MG519784) and those previously reported from loggerhead sea turtle *Caretta caretta* in Cape Verde (FJ948133, AM412641, and DQ535186), black-blotched stingray *Taeniura melanopsila* in Japan (LC019016), and American manatee *Trichechus manatus* in Japan (AB775569). The results indicate that narrow-clawed crayfish can be infected by *A. hydrophila* and *F. solani* simultaneously, and to the best of our knowledge, this is the first report of just such a co-infection in this host. Further studies are necessary to investigate the pathogenicity of these organisms and their effects on narrow-clawed crayfish.

KEY WORDS: Co-infection · *Astacus leptodactylus* · *Aeromonas hydrophila* · *Fusarium solani* · Iran

1. INTRODUCTION

The narrow-clawed crayfish *Astacus leptodactylus* is an important species of crayfish and is reared in numerous countries (SamCookiyaei et al. 2012). In Iran, *A. leptodactylus* is the only species of crayfish and has a limited geographical distribution (Nekuie Fard et al. 2011).

Many pathogens can impact this species in the natural habitat. Two significant genera, *Saprolegnia* and *Aphanomyces* (both from class Oomycota), are believed to infect crayfish (Longshaw 2011). *Aphanomyces astaci* is a water mold that causes crayfish plague, a severe infectious disease. Within the class of Sordariomycetes, a number of fungi have been described in crayfish, which are subsumed under a taxonomic category called *Fusarium*, though these fungi have not been characterized at the species level (Quaglio et al. 2006). The many fungal strains that have been isolated from diseased crayfish as well as other crustaceans include *Aspergillus* spp., *Hormodendrum* spp., *Uncinula* spp., *Alternaria* spp. (Edgerton

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et al. 2002a), and *Fusarium* spp. (Lightner & Fontaine 1975, Chinain & Vey 1987, Colorni 1989, Rhoobunjongde et al. 1991, Khoa et al. 2004, 2005, Khoa & Hatai 2005, Nekuie Fard et al. 2011, Makkonen et al. 2013, Geasa & Naglaa 2014, Tuxbury et al. 2014).

Bacteria can also infect crayfish. These have been found to include the genera *Vibrio*, *Staphylococcus*, *Pseudomonas*, *Micrococcus*, *Flavobacterium*, *Corynebacterium*, *Citrobacter*, *Bacillus*, *Aeromonas*, and *Acinetobacter* (Yahyazadeh et al. 2016).

The infection of a host by 2 or more different pathogens is referred to as co-infection (Kotob et al. 2016). The first case of bacterial–fungal co-infection in *Astacus leptodactylus* (n = 6) was reported by Avsever et al. (2011), who isolated *Saprolegnia* sp. from melanized tissue and *Aeromonas hydrophila* from the hemolymph. The purpose of the present study was to investigate the cause of mortality in diseased narrow-clawed crayfish from Haft Baram Lake, Iran. To the best of our knowledge, this is the first report of *Aeromonas hydrophila* and *Fusarium solani* co-infection in *Astacus leptodactylus.*

2. MATERIALS AND METHODS

2.1. Sampling

A total of 10 moribund narrow-clawed crayfish *Astacus leptodactylus* were taken from Haft Baram Lake (29° 49′ 46″ N, 52° 2′ 34″ E) in Fars province, southern Iran, during spring 2016. The samples were immediately transferred to the Aquatic Animal Health and Diseases Department, School of Veterinary Medicine, Shiraz University, Shiraz, Iran, in appropriate conditions. The crayfish presented with signs of disease, including melanized lesions and abscesses in the cuticle (Fig. 1).

2.2. Microbiological tests

Samples of fluid from inside the intact abscess and melanized lesions in the cuticle were aseptically cultured onto brain heart infusion agar for the culture of bacteria, and Sabouraud dextrose agar for the culture of fungi. Inoculated plates were incubated at 25°C for 48 h for microbiological testing following Austin & Austin (2012) (see our Table 1). PCR was used for the molecular identification of both bacterial and fungal isolates.

2.3. DNA extraction and PCR assay

The genomic DNA of bacteria was extracted using the boiling method (Holmes & Quigley 1981). The genomic DNA of fungal samples were extracted after 3 freeze–thaw cycles, using a commercial DNA extraction kit (Genet Bio, South Korea). Extracted DNA was visualized by agarose gel electrophoresis (0.9%) before being stored at –20°C. Each PCR reaction mix (50 µl) consisted of 4 µl of genomic DNA, 25 µl of 2× Master Mix PCR mixture (Ampliqon, Denmark), 2 µl of each primer (20 pmol), and 17 µl of sterile distilled water. Based on morphology, growth characteristics, and biochemical profiles (Table 1), all bacterial isolates were similar to *Aeromonas hydrophila*. Accordingly, molecular characterization of *A. hydrophila* was conducted based on 16S rDNA markers as described by Dorsch et al. (1994). Primer sequences to identify *A. hydrophila* were 16S-rDNA-F (5′-GAA AGG TTG ATG CCT AAT ACG TA-3′) and 16S-rDNA-R (5′-CGT GCT GGC AAC AAA GGA CAG-3′). For the identification of fungal species, the universal primer pair for the internal transcribed spacer (ITS) was employed; primer sequences were ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4

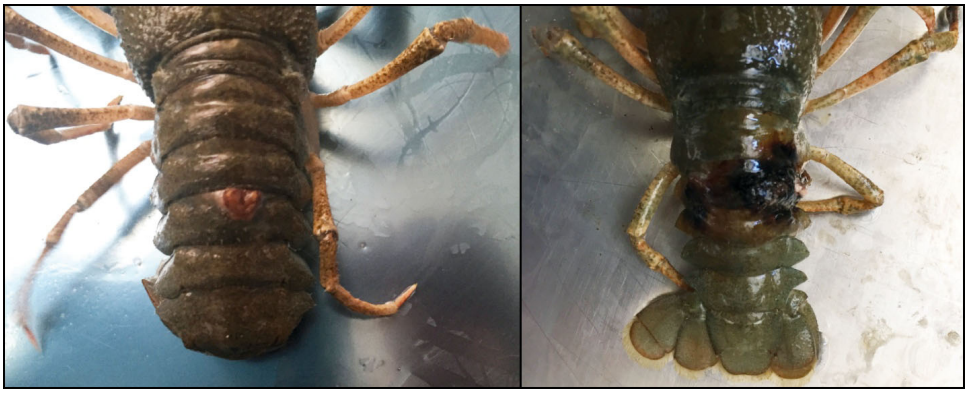

Fig. 1. Two specimens of narrow-clawed crayfish *Astacus leptodactylus* with symptoms of melanized lesions and abscesses in the cuticle

Table 1. Morphology, growth, and biochemical characteristics of bacteria later identified as *Aeromonas hydrophila*, isolated from narrow-clawed crayfish *Astacus leptodactylus*

Test	Result
Gram staining	Negative
Shape	Rod
Oxidase	Positive
Catalase	Positive
Esculin	Positive
Nitrate recovery	Positive
Oxidation/fermentation (O/F)	F
Sulfide indole motility medium	Positive
Indole	Positive
Movement	Positive
Methyl red	Negative
Voges–Proskauer	Positive
Gelatin	Positive
Starch	Positive
Glucose	Positive
Galactose	Positive
Maltose	Positive
Fructose	Positive

(5′-TCC TCC GCT TAT TGA TAT GC-3′). DNA amplification was performed in a thermocycler (MJ Mini, Bio-Rad) under the following conditions: a 5 min initial denaturation at 94°C; followed by 35 cycles at 94°C denaturation for 45 s, 1 min annealing at 55–58°C, 45 s extension at 72°C; and 5 min final extension at 72°C. After the amplification of the target genes, 10 µl of PCR products was subjected to electrophoresis in 1.5% (w/v) agarose gel prepared with 1× Tris-acetate-EDTA buffer and run at 100 V for 45 min. Then, the DNA bands were stained with RedSafe (Intron Biotechnology, South Korea) and viewed on a UV transilluminator. The PCR product sizes were estimated by comparing them with the migration of a 100 bp DNA molecular weight ladder (SinaClon, Iran).

2.4. ITS rDNA sequence analysis

The PCR-amplified products of ITS for the fungal isolate were directly sequenced in both directions on ABI 3730 machines by BigDye terminator sequencing (Applied Biosystems). The forward and reverse directions of the read sequence were used to provide the finalized ITS sequence. The obtained sequence for the ITS was compared to related existing sequences in GenBank using the BLAST tool. Molecular evolutionary analysis was performed using the MEGA 6 program via FASTA algorithms. A maximum likelihood approach was used for the reconstruction of phylogenetic trees (Sneath & Sokal 1973, Tamura et al. 2007).

3. RESULTS

Microbiology specimens were positive in culture for both bacterial and fungal agents. The morphology, growth characteristics, and biochemical tests of the bacterial isolates resembled closely those found in *Aeromonas hydrophila* (Table 1).

In the PCR assay, DNA samples extracted from *A. hydrophila*-like organisms and the fungal samples gave the expected fragment sizes of 685 bp and 600 bp for the 16S rDNA and ITS rDNA regions, respectively (Figs. 2 & 3). The 685 bp and 600 bp bands were not observed in the negative-control lanes (distilled water) in the electrophoresis. PCR products of 16S rDNA were specific for *A. hydrophila* (Dorsch et al. 1994). For the fungal samples, the PCR products produced with the universal primer ITS were sequenced for accurate identification of *Fusarium* (Khoa et al. 2005, Sarmiento-Ramírez et al. 2010).

The fungal isolates were subjected to ITS rDNA sequence analysis and identified as *Fusarium solani*. These strains shared 100% ITS rDNA gene sequence similarity across all our samples. The sequencing result of the amplified products obtained in the pres-

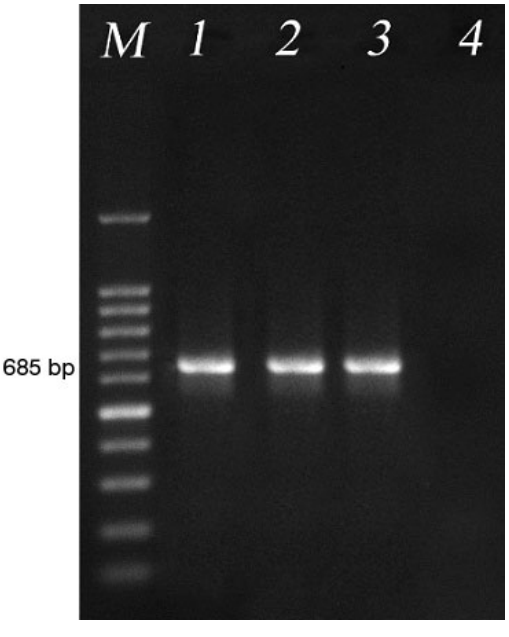

Fig. 2. Electrophoresis image from PCR amplification of 16S rDNA region of *Aeromonas hydrophila*. Lane M: molecular marker of 100 bp; Lane 1: positive control; Lanes 2 and 3: bacterial samples; Lane 4: negative control

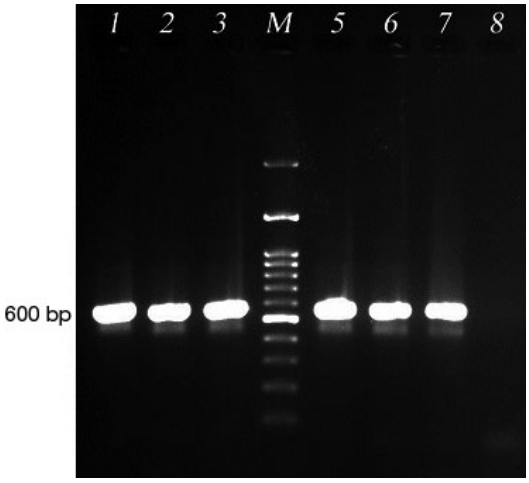

Fig. 3. Electrophoresis image from amplification of ITS rDNA of *Fusarium* sp. Lane M: molecular marker of 100 bp; Lanes 1–3 and 5–7: fungal samples; Lane 8: negative control

ent study has been deposited as an Iranian strain in GenBank (accession number MG519784). Partial sequence analysis of this sequence revealed 100% to 95.86% homology with other available sequences for *F. solani* in GenBank, ranging from 100% similarity to accession numbers LC019016 (host: *Taeniura melanopsila*, Japan) and DQ535186, AM412641, and FJ948133 (host: *Caretta caretta*, Cape Verde), to 95.86% similarity to AY633744 (host: *Marsupenaeus japonicus*, Japan) (Figs. 4 & 5).

The phylogenetic tree constructed using the maximum composite likelihood method is shown in Fig. 4. All the results are based on pairwise analysis of the 11 sequences shown in Fig. 5. In addition, the ITS sequence alignment and comparison showed that intraspecific variations within the population of *F. solani* were detected at several positions (Fig. 6).

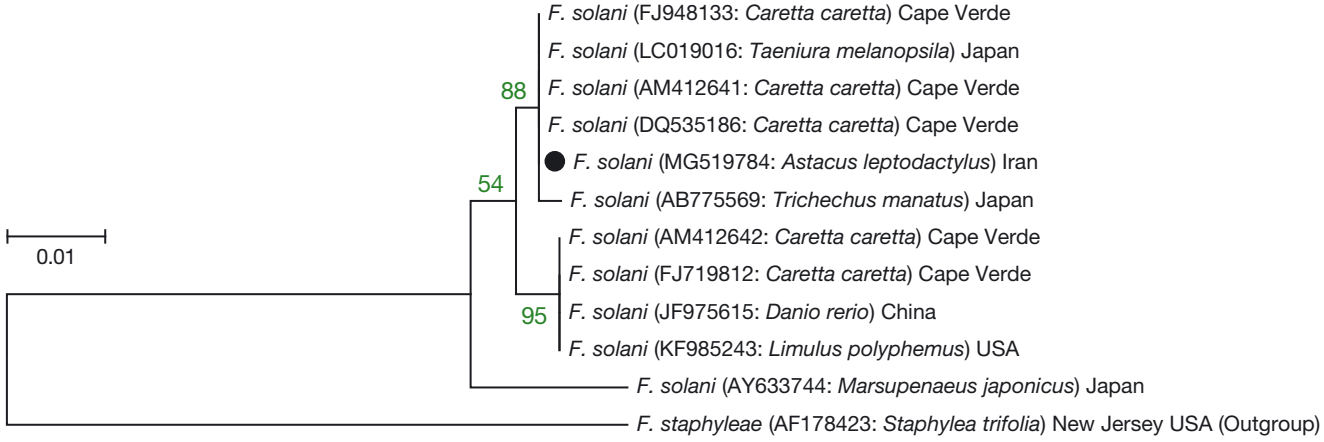

Fig. 4. Molecular phylogenetic analysis of the Iranian isolate *Fusarium solani* obtained from narrow-clawed crayfish *Astacus leptodactylus* in the present study (●) and other related sequences in GenBank based on partial ITS rDNA sequences. Molecular evolutionary analysis conducted using maximum likelihood method in MEGA 6.0. Scalebar shows nucleotide substitutions; green numbers at nodes are bootstrap values for 10 000 replications

		1	2	3	4	5	6	7	8	9	10	11
Iranian isolate	1		1	0	0	0	0	9	9	8	11	19
F. solani (AB775569: Trichechus manatus) Japan	2	99.78		1	1	1	1	10	10	9	12	20
F. solani (DQ535186: Caretta caretta) Cape Verde	3	100.00	99.78		0	0	0	9	9	8	11	19
F. solani (AM412641: Caretta caretta) Cape Verde	4	100.00	99.78	100.00		0	0	9	9	8	11	19
F. solani (FJ948133: Caretta caretta) Cape Verde	5	100.00	99.78	100.00	100.00		0	9	9	8	11	19
F. solani (LC019016: Taeniura melanopsila) Japan	6	100.00	99.78	100.00	100.00	100.00		9	9	8	11	19
F. solani (AM412642: Caretta caretta) Cape Verde	7	98.04	97.82	98.04	98.04	98.04	98.04		0	1	2	18
F. solani (JF975615: Danio rerio) China	8	98.04	97.82	98.04	98.04	98.04	98.04	100.00		1	2	18
F. solani (FJ719812: Caretta caretta) Cape Verde	9	98.26	98.04	98.26	98.26	98.26	98.26	99.78	99.78		3	18
F. solani (KF985243: Limulus polyphemus) USA	10	97.60	97.39	97.60	97.60	97.60	97.60	99.56	99.56	99.34		20
F. solani (AY633744: Marsupenaeus japonicus) Japan	11	95.86	95.64	95.86	95.86	95.86	95.86	96.08	96.08	96.08	95.64	

Fig. 5. Estimation of ITS rDNA nucleotide difference (upper right) and percent identity (lower left) for *Fusarium solani* (in parentheses: GenBank accession number and host) using CLC Main Workbench 5 (CLC Bio)

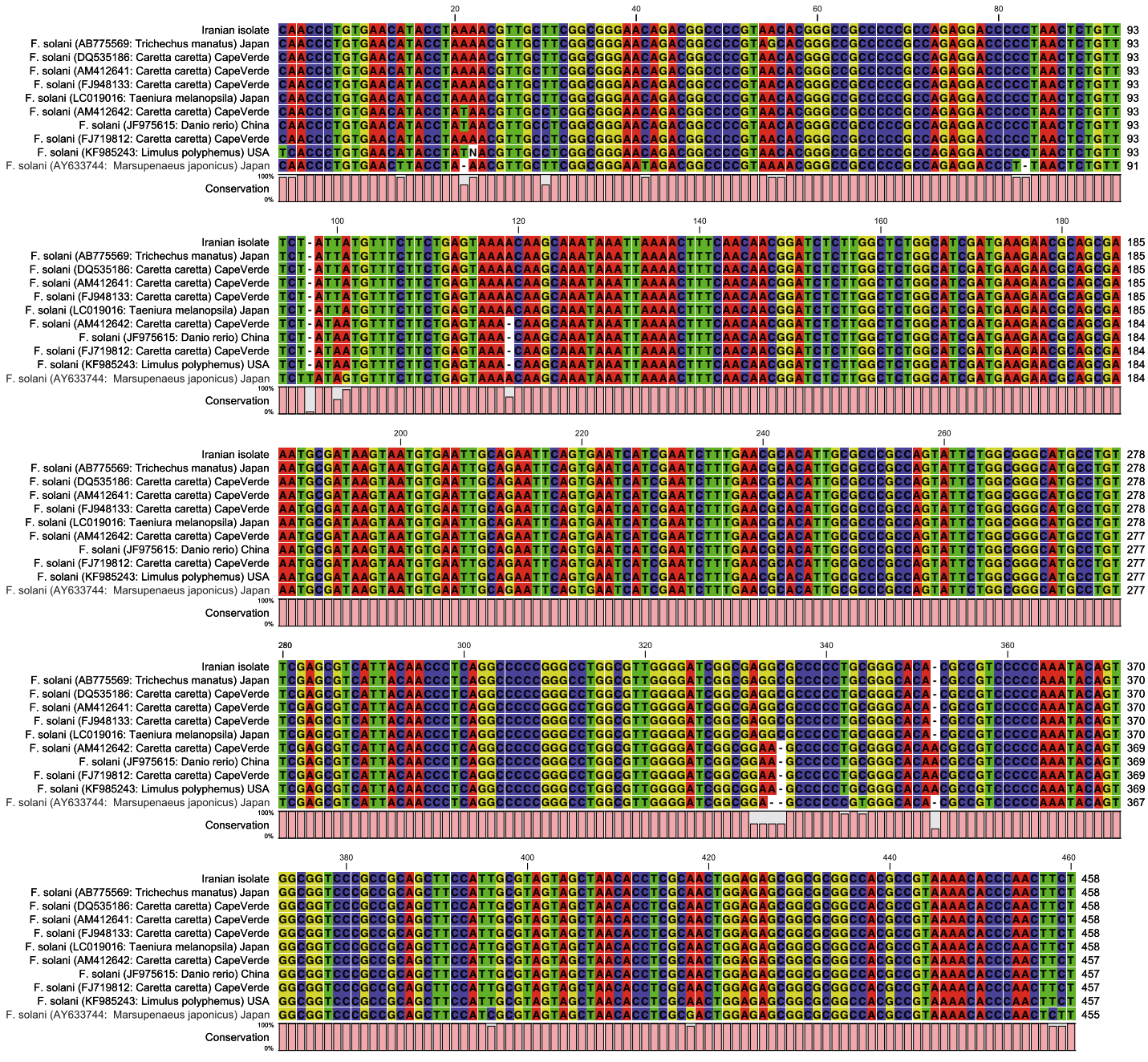

Fig. 6. Alignment of partial nucleotide sequences of ITS rDNA region between our Iranian strain of *Fusarium solani* and other related sequences in GenBank

4. DISCUSSION

The fungal pathogen *Fusarium solani* was found in abdominal lesions of the narrow-clawed crayfish. Identification of fungi may prove to be difficult if done only by morphology. In the present study, the identification of *F. solani* was confirmed by both fungal culture and ITS rDNA sequencing, similar to Khoa et al. (2005) and Tuxbury et al. (2014).

There was a significant level of genetic similarity (varying from 95.86 to 100%) between the present ITS rDNA sequence and those reported from previously recognized *F. solani* in GenBank. Phylogenetic analysis of the molecular data indicated that the present *Fusarium* strain isolated from *Astacus leptodactylus* (Iran: GenBank accession number MG 519784) was genetically related to fungal agents isolated from *Caretta caretta* (Cape Verde: DQ535186,

AM412641, and FJ948133), *Taeniura melanopsila* (Japan: LC019016), and *Trichechus manatus* (Japan: AB775569). These molecular findings are indicative of a common ancestor for *Fusarium* infection in these regions. The Iranian isolate displayed a noticeable diversity with other *F. solani* strains isolated from *Caretta caretta* (Cape Verde), *Danio rerio* (China), *Limulus polyphemus* (USA), and *Marsupenaeus japonicus* (Japan) (Fig. 4).

Findings obtained from phylogenetic analysis suggest that *Fusarium* infections, which affect aquatic species around the world, could be caused by various, and not the same, sources of infection. Therefore, based on the results from microbiology and the data analyzed in the present study, the conclusion could be drawn that the phenotype and genetic characteristics among *F. solani* isolates are considerably diverse and this diversity could be attributed to the host origin or geographical location.

Fungal organisms inhabit and proliferate in a number of places, including in water, substrate, and other animals near the host (Tuxbury et al. 2014). One possible route for fungi to enter a host animal is through previously acquired physical injuries to the carapace and/or abdominal segments. Other factors may include secondary immunosuppression due to stress resulting from captivity and/or other environmental parameters that could have made them susceptible to disseminated fusariosis (Nolan & Smith 2009).

Bacterial infestation in freshwater crayfish is common, and different species of both Gram-negative and Gram-positive bacteria have been isolated from the hemolymph of apparently healthy freshwater crayfish without any clinical signs (Yahyazadeh et al. 2016). The most common include *Aeromonas* spp., *Pseudomonas* spp., *Acinetobacter* spp., *Flavobacterium* spp., *Vibrio* spp., *Citrobacter* spp., *Staphylococcus* spp., *Micrococcus* spp., and *Bacillus* spp. The etiology and pathologic importance of most of them is unknown (Edgerton et al. 2002b). Gram-negative rod bacteria have been found to be prevalent in freshwater crayfish; in the hosts *Astacus astacus*, *Cherax quadricarinatus*, and *C. albidus destructor*, 50%, 35%, and 77% of the observed bacteria were Gram-negative rods, respectively (Edgerton et al. 1995). In the present study, the Gram-negative *Aeromonas hydrophila* was isolated from lesions and abscesses in the cuticle of *Astacus leptodactylus* with some clinical signs. Although the cause of bacterial infestation in apparently healthy freshwater crayfish is not clear, it seems that the presence of bacteria in aquatic environments and environmental stress are the predominant factors for this condition. Madetoja & Jussila (1996) documented the presence of the bacterial infestation incidence in healthy cultured freshwater crayfish and found that the infestation intensified in unsuitable conditions such as high temperature, low dissolved oxygen, and long-term maintenance of harmful conditions.

Aeromonas hydrophila is a ubiquitous Gram-negative bacterium in aquatic environments that is often isolated from apparently healthy crayfish hemolymph or moribund fish and crustaceans (Scott & Thune 1986, Sung et al. 2000, Nielsen et al. 2001, Edgerton et al. 2002b, Jiravanichpaisal et al. 2009, Raissy et al. 2013, Yahyazadeh et al. 2016). However, it has the potential to cause disease in freshwater crayfish, especially in cultured and unsuitable environments (Quaglio et al. 2006). *A. hydrophila* and some other bacteria were isolated from experimentally re-infected *Pacifastacus leniusculus*; the highest mortality rate was due to *A. hydrophila*, which occurred at 22°C and 6 h after the bacterial injection (Jiravanichpaisal et al. 2009). Bacteria may enter the host via the environment, injury, gastrointestinal apparatus, and the hemolymph; subsequently, the host crayfish may act as a carrier and reservoir of the bacterium, which could then become pathogenic in case of unsuitable environmental conditions, molting, stress, and immunodeficiency. If that happens, it could pose a serious threat to freshwater crayfish (Yahyazadeh et al. 2016).

This study is the first report of co-infection of *Aeromonas hydrophila* and *Fusarium solani* in narrow-clawed crayfish. Additional studies are needed to evaluate the pathogenicity of these organisms and their effects on narrow-clawed crayfish.

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