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Aphanomyces astaci genotypes involved in recent crayfish plague outbreaks in central Italy

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ABSTRACT: The oomycete *Aphanomyces astaci* is the causative agent of crayfish plague in native European freshwater crayfish. Molecular analyses showed that several distinct genotype groups of this pathogen, apparently associated with different original host taxa, are present in Europe. Tracking their distribution may contribute to understanding the introduction pathways of *A. astaci*. We used microsatellite markers to genotype the pathogen strains involved in 7 mass mortalities of the endangered indigenous crayfish *Austropotamobius pallipes* that occurred between 2009 and 2016 in the Abruzzi and Molise regions, central Italy. Three *A. astaci* genotype groups (A, B, and D, with the latter represented by 2 distinct multilocus genotypes) were identified, suggesting the existence of multiple infection sources even in a relatively small area. Most crayfish plague episodes were due to genotype groups associated with the North American host species *Pacifastacus leniusculus* and *Procambarus clarkii*, although these crayfish are not widespread in the study area. *A. astaci* genotype group A was detected not only in crayfish plague outbreaks but also in apparently healthy *Astacus leptodactylus* imported for human consumption from Armenia and kept alive in an aquaculture facility. Imports of chronically infected *A. leptodactylus* from Armenia, Turkey, and possibly Eastern Europe are an underestimated introduction pathway for *A. astaci*. Although we cannot exclude the presence of latently infected native populations of *A. pallipes* in the region, *A. astaci* infections in legally imported crayfish species considered vulnerable to crayfish plague may represent further reservoirs of *A. astaci*; this should be reflected in the policies regulating the trade of live crayfish.

KEY WORDS: Invasive crayfish · *Aphanomyces astaci* · Microsatellite genotyping · Crayfish plague · Disease control · *Austropotamobius pallipes* · Italy

INTRODUCTION

The oomycete *Aphanomyces astaci* is the causative agent of crayfish plague, an acute disease of freshwater crayfish that has severely affected populations of indigenous crayfish species throughout the European continent (Alderman 1996, Holdich et al. 2009, OIE 2017) and also endangers crayfish in other parts of the world (Martín-Torrijos et al. 2018, Putra et al. 2018). Its transmission has been primarily facilitated by human activities that involve trade and utilization of crayfish, both for consumption and more recently also for ornamental purposes, and particularly by the spread of invasive species of North American origin that are the natural hosts of *A. astaci* (Holdich et al. 2009, Svoboda et al. 2017). The first wave of crayfish mortalities in Europe caused by crayfish plague had spread only through indigenous crayfish populations (Alderman 1996). However, the original hosts of *A. astaci*, such as *Pacifastacus leniusculus*, *Procambarus clarkii*, and *Orconectes limosus*, which act as chronic carriers thanks to their high resistance to the detri-

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mental effects of infection by this pathogen (Jussila et al. 2015, OIE 2017, Svoboda et al. 2017), were later introduced to Europe to compensate for losses of native crayfish species, and are at present widely distributed across the continent (Holdich et al. 2009, Kouba et al. 2014).

The application of molecular typing techniques to *A. astaci* strains allowed identification of distinct *A. astaci* genotype groups, thus contributing to a better understanding of the relationship between the pathogen and its host taxa (Jussila et al. 2015, Svoboda et al. 2017). The techniques used thus far on *A. astaci* include random amplified polymorphic DNA (RAPD) analysis (Huang et al. 1994, Diéguez-Uribeondo et al. 1995, Kozubíková et al. 2011), amplified fragment length polymorphism (AFLP; Rezinciuc et al. 2014), microsatellite genotyping (Grandjean et al. 2014), and sequencing of the chitinase genes (Makkonen et al. 2012a) and mitochondrial ribosomal markers (Makkonen et al. 2018). The microsatellite- and sequencing-based methods have a substantial methodological advantage, as they are suitable for processing genomic DNA samples extracted directly from the infected tissues of field-collected crayfish, thus avoiding the isolation of *A. astaci* axenic cultures necessary for RAPD- or AFLP-based techniques. Furthermore, the variation in microsatellite markers is sufficient to differentiate among all *A. astaci* genotype groups identified to date and has also revealed some degree of variability within them (Grandjean et al. 2014, James et al. 2017, Mrugała et al. 2017). Therefore, the method allows for retrospective analyses of the disease natural history, pathogen sources, and most likely introduction pathways, based on historical samples preserved from past crayfish plague outbreaks (Grandjean et al. 2014, Vrålstad et al. 2014). For such purpose, the combination of sequences of multiple mitochondrial and nuclear markers (Makkonen et al. 2012a, 2018) may be useful as well.

To date, 5 distinct *A. astaci* genotype groups differing in their RAPD profiles, labeled alphabetically from A to E, have been distinguished (see reviews by Rezinciuc et al. 2015, Svoboda et al. 2017). *A. astaci* strains belonging to group A have been detected in all widespread European crayfish taxa, i.e. *Astacus astacus* (Viljamaa-Dirks et al. 2013, Kozubíková-Balcarová et al. 2014, Vrålstad et al. 2014, Maguire et al. 2016), *Austropotamobius torrentium* (Maguire et al. 2016, Jussila et al. 2017), *Austropotamobius pallipes* (Manfrin & Pretto 2014), and *Astacus leptodactylus* (Huang et al. 1994).

Note that a recent taxonomical revision by Crandall & De Grave (2017) has followed the proposal of Śmietana et al. (2006) to recognize the genus *Pontastacus*, into which *A. leptodactylus* should be assigned. Similarly, the subgenus to which *O. limosus* belongs has been elevated to the genus rank, and the species is referred to as *Faxonius limosus*. In the present contribution, oriented towards readers focusing on diseases of aquatic organisms, we conservatively retain use of the widespread names.

The genotype group A of *A. astaci* was probably the first one introduced to Europe in the 19th century (Huang et al. 1994), and its original American crayfish host species is still unknown. Besides episodes of crayfish plague outbreaks (e.g. Kozubíková-Balcarová et al. 2014), the involvement of *A. astaci* group A strains in latent infections has been demonstrated in all of the above-mentioned host species (reviewed by Svoboda et al. 2017), suggesting possible adaptations towards a lower virulence in these pathogen strains (Jussila et al. 2015).

The remaining known *A. astaci* genotype groups are associated with different American crayfish hosts. Group B comprises strains apparently originating from the signal crayfish *P. leniusculus* (Huang et al. 1994). It has been documented from crayfish plague outbreaks involving indigenous European species in Germany (Oidtmann et al. 1999), Finland (Viljamaa-Dirks et al. 2013), the Czech Republic (Kozubíková-Balcarová et al. 2014), Norway (Vrålstad et al. 2014), France (Grandjean et al. 2014, Collas et al. 2016), and Croatia (Maguire et al. 2016). Furthermore, it has also been reported from latent *A. astaci* infections in some populations of *A. leptodactylus* in Turkey (Svoboda et al. 2014, Kokko et al. 2018) and Eastern Europe (Panteleit et al. 2018). Group C has also been isolated from *P. leniusculus* (Huang et al. 1994), but so far its presence has not been confirmed in Europe. Group D has been isolated from the red swamp crayfish *P. clarkii* (Diéguez-Uribeondo et al. 1995) and was confirmed in crayfish plague outbreaks in *A. pallipes* populations in Spain (Grandjean et al. 2014, Rezinciuc et al. 2014) and France (Grandjean et al. 2014). Furthermore, it was apparently involved in mass mortalities of the indigenous crayfish *Cambaroides japonicus* in Japan (Martín-Torrijos et al. 2018). Finally, group E was described from the spiny-cheek crayfish *O. limosus* (Kozubíková et al. 2011) and was detected in mass mortalities of *A. astacus* and *A. torrentium* in the Czech Republic (Kozubíková-Balcarová et al. 2014) and of *A. pallipes* in France (Grandjean et al. 2014).

Although the application of molecular typing techniques to *A. astaci* may contribute to a better under-

standing of crayfish plague sources and epidemiology (Grandjean et al. 2014, Kozubíková-Balcarová et al. 2014, Svoboda et al. 2017), records on the involvement of the different genotype groups in crayfish plague outbreaks are still limited to the few countries listed above.

In Italy, the most widespread indigenous crayfish species is by far the white-clawed crayfish *A. pallipes* (Aquiloni et al. 2010, Kouba et al. 2014). However, this species is at serious risk of decline or even of local extinctions due to habitat losses and other anthropogenic impacts, as well as due to the widespread presence of several non-indigenous crayfish species. These include mainly *P. clarkii* and, to a lesser extent, *O. limosus* and *P. leniusculus*. *A. leptodactylus* has also been reported, as have other newly established alien species, such as *Cherax destructor*, *C. quadricarinatus*, and *Procambarus virginalis* (Aquiloni et al. 2010, Kouba et al. 2014, Vojkovská et al. 2014).

The white-clawed crayfish is particularly abundant in the watersheds of the central Apennine Mountains located in the Abruzzi and Molise regions (Aquiloni et al. 2010, Caprioli et al. 2014). In this area, *A. pallipes* fisheries have traditionally represented an important economic resource, with large amounts of crayfish regularly delivered to the fish markets of larger cities in central Italy (Vinciguerra 1899, Aquiloni et al. 2010). Since the 1970s, however, the increased impact of anthropogenic activities and crayfish mass mortalities, likely due to the crayfish plague (Aquiloni et al. 2010), led to a drastic decline of *A. pallipes* populations in the lower reaches of watercourses in this area. Consequently, the presence of this crayfish species was confined to the highest reaches and small tributaries, leading to the shutdown of the crayfish trade in the Abruzzi and Molise regions. In the 1990s, *A. pallipes* was included in the wildlife conservation and management programs of these 2 Italian regions as a protected species (Regione Abruzzo 1993), in compliance with the Council Directive 92/43/EEC on the conservation of natural habitats and of wild fauna and flora, and its catch and commercialization were prohibited. In recent years, the import of *A. leptodactylus* from EU countries such as Greece and Bulgaria, as well as from non-EU countries such as Armenia (European Commission 2003), and their sale to restaurants or private citizens have become a common practice.

Several crayfish plague outbreaks that occurred in *A. pallipes* populations in the Abruzzi and Molise regions have been investigated in recent years (Cammà et al. 2010, Caprioli et al. 2013), even though their sources could not be identified. Interestingly, one of these outbreaks occurred only in a restricted area of the involved river basin and appeared to be self-limiting (Caprioli et al. 2013), while in other episodes entire crayfish populations were wiped out. These different disease patterns suggest that *A. astaci* strains of different virulence might have been involved.

For all of these characteristics, the above-mentioned regions may represent a suitable area to investigate the patterns of transmission of crayfish plague. Therefore, the aim of our study was to apply a molecular epidemiology approach to investigate retrospectively the *A. pallipes* mass mortalities that occurred between 2009 and 2016 in the watersheds of the Abruzzi and Molise regions. The *A. astaci* genotypes involved in these events were identified using 9 polymorphic microsatellite markers (Grandjean et al. 2014). We hypothesized that we would detect at least 2 different strains, one more virulent (most likely related to the presence of *P. clarkii* in the region), and another less virulent, involved in the above-mentioned self-limiting crayfish plague outbreak (Caprioli et al. 2013). Furthermore, in the framework of a survey on the presence of non-indigenous crayfish in the Abruzzi region, we investigated the potential role of apparently healthy *A. leptodactylus* individuals, legally imported to Italy for commercial purposes from 2 different source regions and kept alive in aquaculture facilities, as vectors of *A. astaci.*

MATERIALS AND METHODS

Survey on the presence of non-indigenous crayfish in the Abruzzi region

The survey was conducted through a bibliographic search, by scanning internet resources, through personal communications, and during fieldwork. Fieldwork was undertaken between June and October 2016 and covered a total of 68 sites (river stretches as well as natural and private ponds) in the Abruzzi region that have never been investigated before for the presence of crayfish. Monitoring was carried out as previously described (Caprioli et al. 2013, 2014). Briefly, crayfish were collected either by hand or baited traps, and morphologically determined to the species level. In addition, aquaculture facilities primarily focusing on trout production but also trading other aquatic animal products were investigated for the presence of *Astacus leptodactylus* or any other crayfish imported from foreign sources and kept alive for sale for direct human consumption.

Laboratory diagnosis of *Aphanomyces astaci* infection

Procambarus clarkii and *A. leptodactylus* individuals, collected respectively from an agriculture pond and an aquaculture facility (Fig. 1), were brought to the laboratory in insulated containers for subsequent molecular analysis of the presence of *A. astaci* in their tissues. Crayfish sampling and manipulation were carried out according to the Italian legislation complying with EU Regulation No. 1143/2014 on the prevention and management of the introduction and spread of invasive alien species, and with the Council Directive 86/609/EEC on the protection of animals used for experimental and other scientific purposes, respectively. Laboratory diagnosis of *A. astaci* infection was carried out by species-specific detection of the pathogen DNA (a fragment of the nuclear ribosomal internal transcribed spacer region) in DNA samples isolated from the crayfish soft abdominal cuticle. DNA was isolated with the Maxwell16 Tissue DNA Purification Kit (Promega) following the manufacturer's guidelines. The presence of *A. astaci* DNA was tested using the TaqMan minor groove binder quantitative PCR (qPCR) assay developed by Vrålstad et al. (2009), with slight modifications as previously described by Caprioli et al. (2013). A standard curve was generated in each qPCR run with the use of 4 *A. astaci* calibrants. Subsequently, based on the strength of the PCR signal, semi-quantitative agent levels were assigned to each sample according to Vrålstad et al. (2009). *A. astaci*-positive DNA samples were stored at –20°C.

Aphanomyces astaci genotyping

For the identification of *A. astaci* genotype groups, 2 sets of samples were analyzed: (1) *A. astaci*-positive

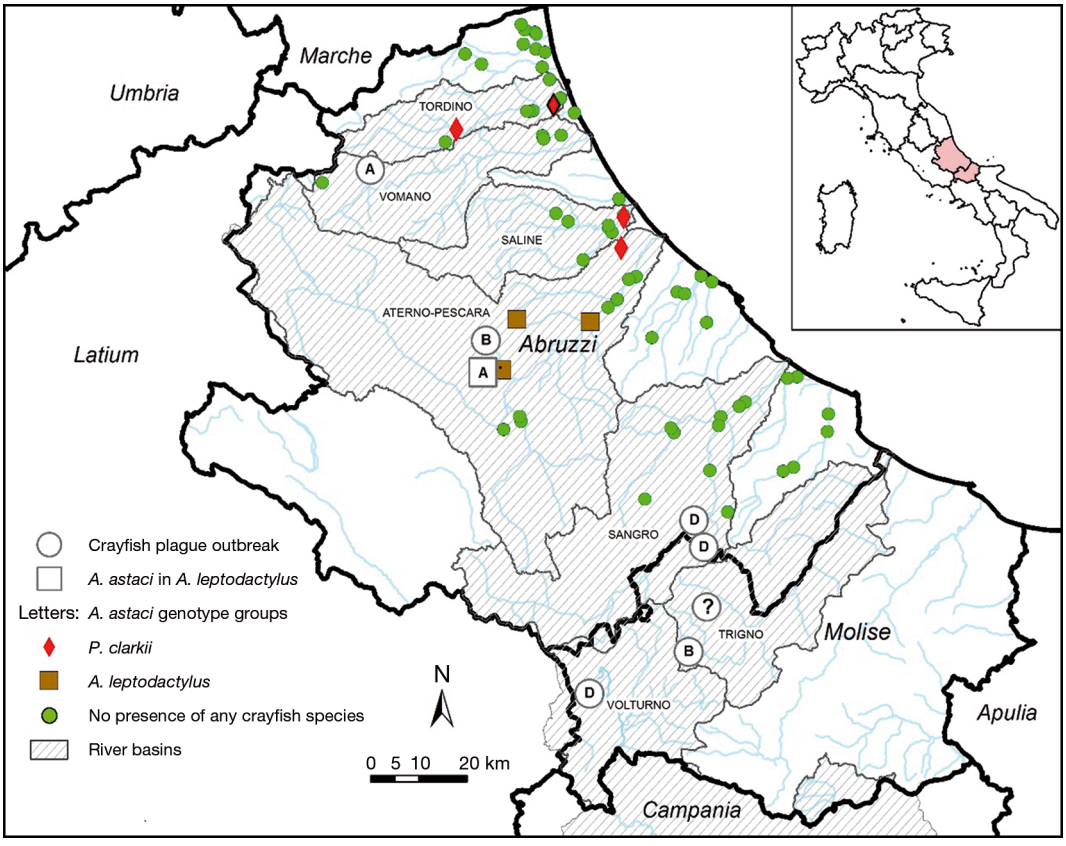

Fig. 1. Distribution of the crayfish plague outbreaks affecting *Austropotamobius pallipes* that occurred in the Abruzzi and Molise regions, Italy, from 2009 to 2016. The map also shows the occurrence of non-indigenous freshwater crayfish in the Abruzzi region, based on the results of a survey conducted between June and October 2016. Outbreaks are indicated by large circles, while the large square indicates the facility where *Aphanomyces astaci*-positive *Astacus leptodactylus* individuals were kept. *A. astaci* genotype groups (A, B, D) are indicated within the symbols (?: genotype not determined). The red diamond with the thicker outline indicates the site where *Procambarus clarkii* individuals were collected and tested for *A. astaci.* Brooks and rivers are indicated in light blue, the region borders with black lines. Striped areas indicate the river basins with crayfish plague outbreaks and/or presence of non-indigenous crayfish

historical DNA samples derived from the tissues of *Austropotamobius pallipes* individuals involved in 7 crayfish plague outbreaks that occurred in river basins of the Abruzzi and Molise regions between 2009 and 2016. The outbreaks occurred either in wild populations living in small mountain watercourses or in experimental hatcheries for restocking purposes, and involved 5 different watersheds (Table 1, Fig. 1). The procedures for outbreak investigations and sampling of dead or dying crayfish have been reported in Caprioli et al. (2013). (2) *A. astaci*-positive DNA samples obtained from *A. leptodactylus* individuals imported for direct human consumption and collected during the survey in the Abruzzi region in 2016 from a trout aquaculture facility located close to the Tirino River (Fig. 1) and releasing effluents into this river. We attempted to score the allele composition at 9 microsatellite markers (Grandjean et al. 2014) for DNA samples with the agent levels A3 or higher, as amplification success strongly depends on the amount of pathogen DNA in the sample (Grandjean et al. 2014).

RESULTS

Distribution of non-indigenous crayfish species in the Abruzzi region

The occurrence of non-indigenous crayfish was recorded in 7 of the 68 sites visited in the Abruzzi region (Fig. 1). *Astacus leptodactylus* was observed in 2 private fishing ponds and in 1 village fountain, and specimens of this species were obtained for further analyses from a trout aquaculture facility located close to the Tirino River (Fig. 1). These crayfish, kept alive for sale in 1 of the facility flow-through tanks, originated from 2 batches legally imported from different countries: from Armenia in February 2016 (9 individuals collected) and from Greece in March 2016 (16 individuals).

Procambarus clarkii was found at 4 sites: 2 agriculture ponds, 1 fishing pond, and 1 pond located in a city park. For analyses of *Aphanomyces astaci* presence, we collected 27 individuals of *P. clarkii* from the population of 1 of the agriculture ponds (Fig. 1).

Laboratory diagnosis of *Aphanomyces astaci* infection

The qPCR analysis confirmed the presence of *A. astaci* DNA in 17 historical samples from 7 crayfish plague episodes affecting *A. pallipes* populations (Table 1), with agent levels ranging from A3 to A7 (Table 1). Moreover, the presence of *A. astaci* DNA was unambiguously confirmed in the tissue samples from 7 out of 9 *A. leptodactylus* imported from Armenia (agent levels ranging from A2 to A5), and a trace signal (agent level A1, not considered a reliable detection) was detected in 1 additional analyzed specimen. In contrast, no *A. astaci* DNA was detected in any of the 16 *A. leptodactylus* individuals imported from Greece nor in the 27 *P. clarkii* specimens collected from the agricultural pond.

Aphanomyces astaci genotyping

A. astaci strains involved in *A. pallipes* mortalities were successfully genotyped in 16 DNA samples from all crayfish plague outbreaks except one (the exception being *A. pallipes* from the Gamberale brook episode in 2013, due to an insufficient amount of *A. astaci* DNA necessary for microsatellite analyses), as well as in the *A. leptodactylus* individuals from Armenia. Three different *A. astaci* genotype groups (A, B, and D) were identified (Tables 1 & 2).

A. astaci genotype group A was detected in a crayfish plague episode that occurred in the Vomano watershed (Abruzzi region) in September 2011 (Caprioli et al. 2013). In addition, it was identified in the apparently healthy *A. leptodactylus* individuals of Armenian origin maintained in the aquaculture facility on the Tirino River.

A. astaci of genotype group B was detected in 2 crayfish plague outbreaks. The first occurred in the Trigno watershed (Molise region) in 2009. The second episode of crayfish mass mortality where group B was detected occurred in February 2016 and affected *A. pallipes* broodstock and juveniles maintained in an experimental hatchery for restocking purposes in the Abruzzi region, located on the Tirino River 10 km upstream from the aquaculture facility importing *A. leptodactylus*.

A. astaci belonging to genotype group D was identified in samples from 3 crayfish plague episodes (Table 1). The first outbreak occurred in October 2011 in the Molise region and wiped out the *A. pallipes* population of the Castelnuovo brook, in the Volturno watershed. The other 2 episodes occurred in July 2013 in the Abruzzi region, close to the Molise border, and affected the Rio Verde brook in the Sangro watershed. These 2 episodes were part of the same outbreak, which was first detected as a mass mortality in an *A. pallipes* experimental hatchery for restocking purposes, located immediately upstream

Table 1. Results of *Aphanomyces astaci* microsatellite genotyping in 7 crayfish plague outbreaks in *Austropotamobius pallipes* populations in the Abruzzi and Molise regions, Italy, between 2009 and 2016, and in apparently healthy *Astacus leptodactylus* individuals collected in an aquaculture facility in the same area in 2016. Genotyping was preferably performed on DNA isolates with the highest available agent levels (exceeding A3 whenever possible; see Grandjean et al. 2014); nd: genotype not determined

Affected crayfish species	Month of sampling	Locality (close settlement, region)	Watershed	Geographical coordinates	Background information (setting; number of *A. astaci*-positive samples)	Agent level	*A. astaci* genotype
A. pallipes	September 2009	San Leo brook (Carovilli, Molise)	Trigno	41.6911° N, 14.2938° E	Mass mortality in a wild population; 3 samples[a]	A3–A7	B
A. pallipes	September 2011	Zingano brook (Crognaleto, Abruzzi)	Vomano	42.5836° N, 13.4775° E	Mass mortality in a wild population; 4 samples[b]	A3–A7	A
A. pallipes	October 2011	Castel Nuovo brook (Rocchetta al Volturno, Molise)	Volturno	41.6147° N, 14.0496° E	Mass mortality in a wild population; 2 samples	A4	D
A. pallipes	July 2013	Rio Verde brook (Borrello, Abruzzi)	Sangro	41.9147° N, 14.3232° E	Mass mortality in an experimental hatchery; 2 samples	A6–A7	D
A. pallipes	July 2013	Rio Verde brook (Rosello, Abruzzi)	Sangro	41.9012° N, 14.3288° E	Mass mortality in a wild population; 2 samples	A5–A6	D
A. pallipes	September 2013	Gamberale brook (Agnone, Molise)	Trigno	41.7742° N, 14.3317° E	Mass mortality in a wild population; 2 samples	A3	nd
A. pallipes	February 2016	Tirino River (Capestrano, Abruzzi)	Aterno-Pescara	42.2713° N, 13.7819° E	Mass mortality in an experimental hatchery; 2 samples	A4–A7	B
A. leptodactylus	February 2016	Tirino River (Bussi, Abruzzi)	Aterno-Pescara	42.2175° N, 13.8179° E	Live crayfish intended for human consumption; 8 samples	A2–A5	A

[a]Described by Cammà et al. (2010); [b]Described by Caprioli et al. (2013)

Table 2. Results of microsatellite genotyping of *Aphanomyces astaci*-positive DNA samples from the tissues of *Austropotamobius pallipes* individuals involved in crayfish plague outbreaks and imported *Astacus leptodactylus* individuals. Fragment sizes for reference strains of *A. astaci* genotype groups A–E (based on Grandjean at al. 2014) are provided for comparison. References are abbreviated as follows: D95: Diéguez-Uribeondo et al. (1995); H94: Huang et al. (1994); K11: Kozubíková et al. (2011)

Genotype	*A. astaci* strain	Host species	Origin & reference/ Locality	Fragment sizes at microsatellite loci (bp)								
				Aast-2	Aast-4	Aast-6	Aast-7	Aast-9	Aast-10	Aast-12	Aast-13	Aast-14
Reference strains												
A	VI03557[a]	*A. astacus*	Sweden (1962); H94	160	103	157	207	180	142	–	194	246
B	VI03555[a]	*P. leniusculus*	USA (1970); H94	142	87	148	215	164/182	132	226/240	202	248
C	VI03558[a]	*P. leniusculus*	Sweden (1978); H94	154	87	148	191	164/168	132	226	202	248
D	VI03556[a]	*P. clarkii*	Spain (1992); D95	138	131	148	203	180	142	234	194	250
E	Evira4805[b]	*O. limosus*	Czech Republic (2010); K11	150	87/89	148/157	207	168/182	132/142	240	194/202	248
Crayfish plague outbreaks in Abruzzi and Molise regions												
A		*A. pallipes*	Zingano brook, 2011	160	103	157	207	180	142	–	194	246
B		*A. pallipes*	San Leo brook, 2009	142	87	148	215	164/182	132	226/240	202	248
B		*A. pallipes*	Tirino River, 2016	142	87	148	215	164/182	132	226/240	202	248
D		*A. pallipes*	Rio Verde, hatchery, 2013	138/182	131	148	203	180	142	234	194	250
D		*A. pallipes*	Rio Verde brook, 2013	138/182	131	148	203	180	142	234	194	250
D		*A. pallipes*	Castel Nuovo brook, 2011	138	131	148	203	180	142	234	194	250
***A. astaci* infection in crayfish imported from Armenia**												
A		*A. leptodactylus*	Aquaculture, 2016	160	103	157	207	180	142	–	194	246

[a]VI numbers refer to assigned strain numbers in the culture collection of the Norwegian Veterinary Institute, Oslo, where the isolates are maintained. Original codes for reference strains VI03557 (A), VI03555 (B), VI03558 (C), and VI03556 (D) are L1, P1, Kv, and Pc, respectively (Huang et al. 1994, Diéguez-Uribeondo et al. 1995)

[b]Evira number refers to a strain in the culture collection of the Finnish Food Safety Authority Evira (OIE reference laboratory for crayfish plague), Kuopio

to the Borrello waterfall barrier and supplied with running water from the adjacent Rio Verde brook. Later, the wild *A. pallipes* population of the brook, already present only upstream of the Borrello waterfall barrier, was wiped out.

The multilocus genotyping patterns corresponded to those of the reference *A. astaci* axenic cultures described by Grandjean et al. (2014) in most samples, with the exception of those originating from 2 sites of the *A. pallipes* mass mortality in the Rio Verde brook (Table 2). In those samples, all markers but one matched the reference axenic culture of the genotype group D, but a consistent difference was observed at the Aast02 locus in all the samples used for genotyping: a heterozygote with allele sizes of 138 and 182 bp was scored instead of the homozygote at 138 bp of the reference strain (Grandjean et al. 2014). To verify these results, the amplification of this marker was successfully repeated in all 4 samples from the Rio Verde brook (Table 1), and heterozygosity at this locus was consistently scored.

DISCUSSION

Several crayfish plague outbreaks have recently affected the *Austropotamobius pallipes* populations in the Abruzzi and Molise regions in central Italy. The genotyping of the *Aphanomyces astaci* strains involved in those outbreaks provides compelling evidence of multiple infection sources even in this relatively small area, where the presence of non-indigenous crayfish had not been recorded until recently. In particular, our findings indicate that the apparent absence of North American crayfish species in a watershed does not exclude the potential involvement of highly virulent *A. astaci* strains in crayfish mortalities. Furthermore, our study highlights that even the legal transboundary import of a crayfish species native to Europe, such as *Astacus leptodactylus*, may represent a relevant introduction pathway for *A. astaci*, and thus a potential infection source. Although *A. leptodactylus* is generally considered susceptible to *A. astaci* (Svoboda et al. 2017), Unestam (1969) already considered it of 'moderate' resistance, and indeed the species has been repeatedly shown to be chronically infected by this pathogen (Kokko et al. 2012, 2018, Pârvulescu et al. 2012, Svoboda et al. 2012, Panteleit et al. 2018). This suggests that *A. leptodactylus* should be considered as a potentially important reservoir of the disease, and treated as such when it is imported, transported, and handled.

The confirmation of *A. astaci* genotype group A as the cause of the *A. pallipes* mortality that occurred in 2011 is consistent with the failure to reveal the presence of non-indigenous crayfish species in the area during the outbreak investigation (Caprioli et al. 2013). It was hypothesized that an *A. astaci* strain characterized by a lower virulence must have been involved, as this apparently self-limiting outbreak affected only a limited area of the river basin (Caprioli et al. 2013). Our findings now support that hypothesis, which is in agreement with the view that strains of genotype group A have adapted to some extent to co-exist with the indigenous European crayfish hosts (Jussila et al. 2015). The involvement of strains of this genotype group in chronic infections has been demonstrated multiple times (e.g. Jussila et al. 2011, 2017, Viljamaa-Dirks et al. 2011, 2013, Manfrin & Pretto 2014, Kokko et al. 2018), and their reduced virulence has been confirmed by experimental infections (e.g. Makkonen et al. 2012b, Becking et al. 2015, Mrugała et al. 2016). However, this *A. astaci* genotype group may still cause mass mortalities (e.g. Kozubíková-Balcarová et al. 2014), and it was recently demonstrated that even strains isolated from latently infected *Austropotamobius torrentium* caused high mortality of *Astacus astacus* in an experimental setting (Jussila et al. 2017).

The propensity of *A. astaci* genotype group A to cause latent infections is further confirmed by its detection in apparently healthy *A. leptodactylus* individuals maintained in an aquaculture facility and intended for direct human consumption. Freshwater crayfish represent a traditional food in several areas of the Abruzzi and Molise regions and, after the ban on the catch and commercialization of *A. pallipes*, the import of *A. leptodactylus* from abroad has become common. These crayfish are often maintained in trout aquaculture facilities that may be connected with water bodies hosting *A. pallipes* populations. Unfortunately, our study demonstrates that imported *A. leptodactylus* may be infected by the crayfish plague pathogen, and thus contribute to its spread as well. The occurrence of latent *A. astaci* infections in populations of this host species has already been shown in Turkey (Kokko et al. 2012, 2018, Svoboda et al. 2012), the Danube in Romania (Pârvulescu et al. 2012, Schrimpf et al. 2012), and the Dniester river in Moldova (Panteleit et al. 2018). Interestingly, 2 different *A. astaci* genotype groups, A and B, were documented in these chronic infections (Svoboda et al. 2014, Kokko et al. 2018, Panteleit et al. 2018). In Armenian commercial stocks of *A. leptodactylus*, latent infections might be as frequent as in Turkey,

since a strain of the genotype group A has been isolated from crayfish apparently imported from the same source country to the Czech Republic (Becking et al. 2015). The confirmation of infected crayfish in commercial trade, however, does not imply that this must have been the original source of the pathogen in the 2011 crayfish plague outbreak in the Vomano watershed. Indeed, we cannot exclude the presence of latently infected native populations of *A. pallipes* in the region, as already reported by Manfrin & Pretto (2014) in northern Italy, and further studies are needed to investigate this possibility.

Although the presence of signal crayfish *Pacifastacus leniusculus* has been previously observed in Italy (Aquiloni et al. 2010, Kouba et al. 2014), this species has never been reported in the Abruzzi and Molise regions (Chiesa et al. 2006, this study). The involvement of *A. astaci* genotype group B, of which *P. leniusculus* is the natural host (Huang et al. 1994), in 2 mass mortalities that occurred 7 yr apart in localities that are about 100 km distant from each other might support the hypothesis that some signal crayfish populations are present in the area but have thus far remained undetected (for example, in private ponds or home aquaria) and that the outbreaks have been caused by illegal crayfish release, or by transmission of *A. astaci* zoospores from such sources (e.g. via contaminated fishing gear). Furthermore, the local presence of non-indigenous crayfish that could have been the source of the infection is sometimes confirmed only several years after the respective crayfish plague outbreaks (see case studies in Kozubíková-Balcarová et al. 2014).

An alternative explanation of the origin of genotype group B in the study region would be an import of infected *A. leptodactylus.* As shown both in Turkey and Eastern Europe, this species may be chronically infected by *A. astaci* strains of this group (Svoboda et al. 2014, Kokko et al. 2018, Panteleit et al. 2018). The hatchery where one of the studied crayfish plague outbreaks occurred is supplied with running water from the adjacent Presciano spring of the Tirino River (Caprioli et al. 2014), and is located 10 km upstream of the aquaculture facility where *A. astaci*-positive *A. leptodactylus* individuals (infected by an A-group strain) were maintained at the same time (Fig. 1). The involvement of 2 different genotype groups (Table 2) makes a direct link between those 2 events unlikely; however, a previous import of a batch of *A. leptodactylus* infected by another *A. astaci* genotype cannot be excluded. Finally, the pathogen might be sporadically present in the area even in the absence of the original host, if it maintains itself in the crayfish plague outbreaks and spreads in a stepping stone manner, or if it persists as a chronic infection in some host populations. The connectivity through the river network could then facilitate its spread to more susceptible crayfish populations.

A. astaci of genotype group D, which was involved in crayfish plague outbreaks from 2 different watersheds in 2011 and 2013, are associated with *Procambarus clarkii* (Diéguez-Uribeondo et al. 1995). This is the most successful non-indigenous crayfish species in Italy (Aquiloni et al. 2010, Kouba et al. 2014), and its role as a carrier of *A. astaci* (Aquiloni et al. 2011) and other potentially pathogenic fungi (Dörr et al. 2011) has been already considered for the country. In the study area, our survey showed the presence of *P. clarkii* in 4 confined ponds in the Abruzzi region, while no reports are available for the Molise region. Although its presence in the study area seems to be still limited, it has to be considered that *P. clarkii* is widespread in other regions of central Italy (Aquiloni et al. 2010), and its wide distribution has been described in the Latium region (Chiesa et al. 2006), bordering both Abruzzi and Molise (Fig. 1). An abundant population of the species has also been reported in Trasimeno Lake in the Umbria region (Fig. 1), even though mycological investigations did not report the presence of *A. astaci* in that population (Dörr et al. 2012). Moreover, several unofficial reports of an abundant presence of *P. clarkii* downstream in the Volturno watershed in the Campania region, bordering Molise (Fig. 1), have been posted on the internet by fishermen and environmental protection associations. Altogether, these reports suggest that *P. clarkii* may have been the source of the *A. astaci* genotype group D infections that were involved in *A. pallipes* mortalities in our study area, even though we did not detect *A. astaci* in the single *P. clarkii* population tested in our study, and the ponds with confirmed presence of this species are at least 70 km from the outbreak sites.

The variation observed at 1 of the microsatellite loci suggests that the outbreaks in 2011 and 2013 may have been caused by different *A. astaci* strains, though apparently belonging to the same genotype group D (Table 2). Such within-group variation is not uncommon and has been reported from Croatia (Maguire et al. 2016), Japan (Mrugała et al. 2017), and the UK (James et al. 2017). Similarly, analyses of mitochondrial markers have recently shown that multiple haplotypes can be distinguished within the D-haplogroup (Makkonen et al. 2018, Martín-Torrijos et al. 2018).

Although variation within a single microsatellite locus detected from mixed genome samples and not accompanied by the isolation of axenic culture of the pathogen cannot be considered definite proof of distinctness between the *A. astaci* strains involved, consistent genotyping results obtained from multiple host individuals collected from specific geographical areas (see also James et al. 2017) support the hypothesis that such variation may be useful in the identification of *A. astaci* infection sources.

To sum up, microsatellite genotyping of preserved samples revealed retrospectively that at least 3 different genotype groups (A, B, and D) of *A. astaci* were involved in *A. pallipes* mass mortality events that occurred in the Abruzzi and Molise regions since 2009. Although the presence of North American crayfish species in these 2 regions does not appear to be as widespread as in other European countries (Kouba et al. 2014) and in many other Italian regions (Chiesa et al. 2006, Aquiloni et al. 2010, Dörr et al. 2012), most crayfish plague episodes were apparently caused by the genotype groups originally associated with *P. leniusculus* and *P. clarkii* (Grandjean et al. 2014). It also seems that at least 2 independent transmissions of genotype group D strains have occurred in different outbreaks, and it is noteworthy that *A. astaci* genotype group B strains have caused mass mortalities despite the absence of signal crayfish reports from the area. It is thus important that efforts to limit the spread of North American crayfish remain an integral part of conservation activities.

Finally, we would like to highlight the importance of finding latent *A. astaci* infections in non-American crayfish legally imported for human consumption. While the threat represented by the American crayfish trade is well known (Holdich et al. 2009), the import of species that are native to Europe and are considered susceptible to *A. astaci* is currently not considered a problem. However, latent infections in such indigenous crayfish, especially *A. leptodactylus* that seems to cope particularly well with the infection, may represent a further introduction pathway and reservoir for *A. astaci*, thus contributing to the persistence of the pathogen in European water bodies. According to the European Union legislation (European Commission 2003), import of live *A. leptodactylus* is authorized only for direct consumption, but not for aquaculture purposes. However, as in the case reported herein, these imported crayfish are usually kept alive in aquaculture facilities until sale. Moreover, some of the sold crayfish may be used for purposes other than consumption, such as for release into garden ponds (Patoka et al. 2014), thus aggravating the risk of pathogen spread. Latent infections of imported crayfish species native to Europe and adjacent regions may thus represent a relevant introduction pathway for *A. astaci* to Europe. If kept in open water bodies or released intentionally or accidentally into the wild, these animals may become another reservoir of this pathogen. Based on these findings, the current regulations of the live crayfish trade should be reviewed. In our opinion, sources of imported crayfish could be regularly screened for *A. astaci* presence, and individuals from populations with chronic *A. astaci* infections should not be imported alive. Furthermore, keeping any imported *A. leptodactylus* individuals should not be allowed in facilities that are directly connected with water bodies hosting other native crayfish species, such as *A. pallipes.*

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