Is there a fungal community associated with the freshwater invasive Asiatic clam *Corbicula fluminea* (Müller, 1774)? The case of some populations in the low basin of the Ticino River (Lombardy, Northern Italy)

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ABSTRACT

The freshwater Asiatic clam Corbicula fluminea is one of the most successful introduced bivalves in the world and, due to its high invasiveness; it can also be a potential vector for diseases and parasites, including fungi. With the aim of filling the gap in knowledge of any mycological information regarding this clam, we report the first characterization of the fungal communities associated with three populations of C. fluminea collected from three tributaries of the Ticino River (Lombardy, Northern Italy). By means of a culturable mycological analysis, 110 specimens were investigated and 83.6% of them yielded the isolation of 13 fungal species. Differences in timing of fungal development, distribution of taxa in the basin, abundance and frequency of the fungal community were highlighted. Four species were occasional, with a frequency of less than 5%, while another five species showed a frequency of almost 20%, locally reaching 40%.

In particular, we highlighted: a) the presence of various fungal flora associated to C.fluminea, sometimes with stable colonization; b) the isolation of Geotrichum candidum, shown to be site-specific in one tributary with high abundance and frequency, which is well known for its generic pathogenity and might be related to a valve erosion observed in the associated Asiatic clam population; c) the first report in water ecosystems of Chaetomium nigricolor, which stably colonizes the three investigated populations of the Asiatic clam.

Our results are encouraging for the continuation of research on the ecological role of the fungal communities associated with aquatic organisms.

Keywords: *microfungi, Geotrichum candidum, non-indigenous species, bivalve*

INTRODUCTION

Freshwater ecosystems are extremely vulnerable to aquatic invasions because they are often interconnected by means of natural and artificial networks; further, biological and anthropogenic vectors that are not strictly aquatic can also contribute to the new colonization of small species [1]. In freshwater ecosystems, bivalves are important filter-feeders and they may be responsible for the control of the concentration and composition of suspended particles. Furthermore, bivalves can represent a link between different habitats, functioning as an important transfer route for food, nutrients and energy, also from aquatic to adjacent terrestrial ecosystems [2]. One of the most successfully introduced bivalves in the world is the freshwater Asiatic clam *Corbicula fluminea* (Müller, 1774) (*Veneroida Corbiculidae*). It is native to south-eastern Asia but its current distribution covers almost all continents [3,4]. In Europe, its range has progressively expanded to most European countries (DAISIE – Delivering Alien Invasive Species Inventories for Europe (http://www.europe-aliens.org). *C. fluminea* can survive in altered environmental conditions better than other freshwater molluscs and its tolerance is one of the main reasons for its success. It colonizes the first layer of sandy-gravelly river and irrigation drainage system bottoms, where it can survive for quite a long life span (3 years to 5 years) [5]. The ecological relevance of this invasive bivalve is evident in the trophic chain because it can accumulate contaminants, even at low concentrations in the environment, bio-amplifying them along the food chain with important impacts on higher trophic levels. These changes that occur at the first trophic level may produce a bottom-up effect with important reverberating modifications in all trophic levels [2]. For example, [6] has shown that *C. fluminea* may be

responsible for important changes in the concentration of plankton, inorganic and organic particles including fungal spores, motile suspended matter from the water column to the benthos.

Due to its high invasiveness, *C. fluminea* can also act as a potential vector for parasites, diseases and fungal dispersion in freshwater ecosystems [7]. According to [8], about 600 fungal species ranging from saprotrophs to pathogens of both plants and animals are associated with aquatic ecosystems. Even though information on their diversity, biomass and ecological role is limited and less available compared to the terrestrial habitats, there is evidence to suggest that fungi play an important role in supporting and sustaining aquatic faunal populations. For example, it is known that finfish, crustaceans, corals, marine molluscs, shellfish, turtles, tadpoles and adult anurans can be highly affected by a wide number of mitosporic fungi [9].

The fungal community associated with freshwater molluscs is generally understudied and the only studies performed are related to the species which have some direct economic interests [10].

On the other hand, there is a total lack of mycological information regarding the fungal community associated with the Asiatic clam. Thus, this paper reports the first characterization of the fungal communities associated with some populations of *C. fluminea* collected in the low basin of the Ticino River (Lombardy, Northern Italy). Particular attention has been given to fungi which could adversely impact the Asiatic clam or the organisms that share the same habitat.

MATERIALS AND METHODS

Study area and sample collection

From February to June 2015, specimens of *C. fluminea* were collected monthly by means of a sampling net (1mm mesh) within a 50x25cm frame (area of $0.5m^2$), in three different semi-natural streams in the complex irrigation system of the low basin of the Ticino River near Pavia in Lombardy, Northern Italy (figure 1). The first stream is the Vernavola Stream, which is an important ecological connection between sites of ecological relevance around the urban area of Pavia; the other two streams are the Gaviola Stream and the Castellana Stream, two typical small streams of the Ticino River flood plain, which are surrounded by agricultural fields. Despite this anthropogenic pressure, the bottom of both streams is covered by aquatic macrophytes.
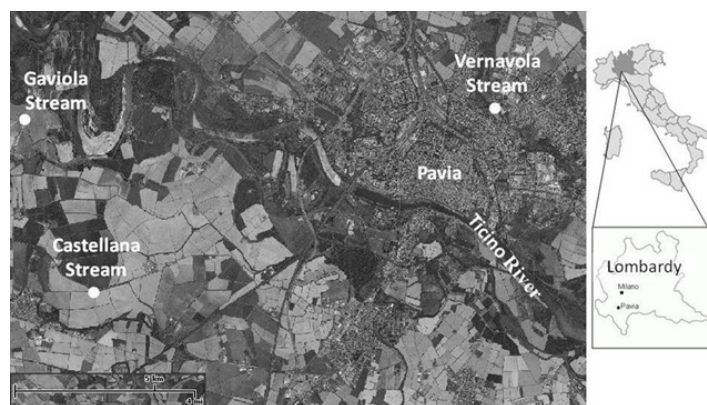

Figure 1: Study area with the localization of the sampling sites in the three streams.

Taxonomic identification of the fungal flora

In order to describe the fungal flora associated with *C.fluminea*, ten specimens were randomly selected from each stream (for a total of 110 specimens) and investigated by means of culturable mycological analysis. As we could not refer to a previously tested technique, the investigation was based on the direct plating method, one of the best ways to study the colonization of a specific substrate, which detects hyphae that have penetrated and grown therein. Such a method has already proved useful for the study of fungal endophytes in vegetal tissues [11], food-borne fungi [12] and fungi on crustaceans [13].

Constantly working under a laminar flow chamber, specimens were first surface-disinfected by being soaked in a sodium hypochlorite solution (0.4%) for 2 minutes, then rinsed with sterile distilled water, and crushed by means of a sterile pestle.

Both small pieces of shells and soft parts of each specimen were plated on Petri plates (1 plate of 9cm diameter/ specimen) containing MEA medium (Malt Extract Agar, medium composition: malt extract powdered 20g, peptone 1g, glucose 1g, agar 15g, distilled water 1000ml). Respectively after 4,6,8 and 10 days of incubation at 25°C, the samples were observed by means of a stereomicroscope (50×) and each developed fungal structure was identified on the basis of macro-morphological analysis of the colonies and microscopical observation of the reproductive structures. Specific taxonomical keys were used for strain characterization [14,15,16,17]. Moreover, for a few cases of dubious classification at species level, DNA sequencing techniques were applied.

As regard to the mycelium characteristics, the growth was obtained with 100 ml PDB (Potato Dextrose Broth, medium composition: infusion from potato 6.5g, dextrose 20g, distilled water 1000ml) or PDA (Potato Dextrose Agar, medium composition: potato 200g, dextrose 20g, agar 15g, distilled water 1000ml). The obtained mycelia were respectively filtered and collected with a sterile spatula, then both frozen, lyophilized and grinded with sterile mortar and pestle.

The resulting mycelium powder (0.02g) was used for DNA extraction by means of a DNeasy Plant Mini Kit (Qiagen, Inc.). The extracted DNA was detected on 1% agarose gel through electrophoresis.

The internal transcribed spacer (ITS) and 5.8 gene of the nuclear ribosomal gene repeat were amplified by polymerase chain reaction (PCR) with the universal primers *ITS1* and *ITS4* using Thermo Scientific DreamTaq Green PCR Master Mix, which contains DreamTaq™ DNA polymerase, DreamTaq Green buffer, MgCl2 and dNTPs [18]. PCR reactions were conducted according to these conditions: 2 minutes at 95°C for initial denaturation followed by 34 cycles (30sec at 95°C for denaturation, 1 minute at 55°C for annealing, 1 minute at 72°C for extension) and 5 minutes at 72°C for terminal extension. Amplified PCR products were detected on 1% agarose gel through electrophoresis.

The PCR products were purified using Applied Biosystem™ CleanSweep™ PCR Purification Reagent and shipped to Macrogen Europe (Macrogen, Inc.) for Sanger Sequencing. The nucleotide sequences obtained were checked against the sequences available in the GenBank database by means of BLAST searches (www.ncbi.nlm.nih.gov).

Statistical analysis of the fungal communities

For each fungal taxon, the development of mycelia was described by means of three qualitative classes (+: low development; ++: medium development; +++: high development). Thereafter, these classes were converted into numerical values (10,50 and 100), and used as estimates of abundance for univariate and multivariate analysis.

Similarities between fungal communities associated with each population of *C. fluminea* were studied by means of SIMPER analysis, in order to identify the taxa which primarily provide the discrimination between samples, and supported by the ANOSIM test, based on the Bray-Curtis similarity matrix. All statistical analysis was performed using PRIMER 6.0.

RESULTS AND DISCUSSION

As a consequence of a flood of the Ticino River, the sample from April 2015 in the Castellana Stream was not collected, therefore a total of 110 specimens (instead of 120) were processed for the investigation of fungal growth: 92 of which (83.6%) yielded the isolation of fungi for a total of 13 species, reported in Table 1.

Table 1: List of the identified fungal species (*confirmed by molecular identification) associated with *Corbicula fluminea* collected in three streams: G-Gaviola; C-Castellana; V-Vernavola

Fungal *taxa*	G	C	V
Acremoniella atra (Corda) Sacc.	x		x
Acremonium strictum W. Gams		x	
Aspergillus flavus Link	x	x	x
Aspergillus niger Tiegh.		x	x
Clonostachys rosea (Link) Schroers, Samuels, Seifert & W. Gams	x		
*Chaetomium nigricolor** L.M. Ames	x	x	x
Fusarium equiseti (Corda) Sacc.			x
Fusarium oxysporum Schltdl.	x	x	
Fusarium verticillioides (Sacc.) Nirenberg			x
*Geotrichum candidum** Link	x		
Mucor hiemalis Wehmer	x		x
Penicillium chrysogenum Thom	x	x	x
Trichoderma harzianum Rifai	x		x
Total species	**9**	**6**	**9**

Three different timings of fungal development were observed: the first to emerge was *Geotrichum sp.* (4 days of incubation) while the last was *Chaetomium sp.* (10 days of incubation). The remaining taxa were detected between the 6th and the 8th day of observation. The isolates of the above-mentioned genera were identified in detail by means of DNA sequencing, and were shown to belong to the species *Geotrichum candidum* [syn. *Dipodascus geotrichum* (E.E. Butler & L.J. Petersen) Arx] and *Chaetomium nigricolor*. Only six out of thirteen species were isolated in the Castellana Stream samples: *Acremomium strictum* was site-specific, while *Aspergillus niger* and *Fusarium oxysporum* were also recorded in the other two streams.

The other two streams hosted a total of nine species each, with four site-specific species: *G. candidum* and *Clonostachys rosea* for the Gaviola Stream, while *Fusarium equiseti* and *Fusarium verticillioides* were only isolated in the Vernavola Stream samples. Finally, *Aspergillus flavus*, *C. nigricolor* and *Penicillum chrysogenum* were found in all three streams (table 1). However, despite these affinities, the Gaviola Stream showed a richer (in terms of number of fungal species), more abundant and more stable fungal community (Table 2, figure 2).

Table 2: Mycelium abundance of the fungal species detected on the specimens of *Corbicula fluminea* from February to June 2015 in the three tributaries of the Ticino River. N.C.: samples not collected

	Gaviola Stream				Castellana Stream				Vernavola Stream			
	Feb	Apr	May	Jun	Feb	Apr	May	Jun	Feb	Apr	May	Jun
Acremoniella atra		+				N.C.				+		
Acremonium strictum						N.C.		++				
Aspergillus flavus	+++				+	N.C.			+			
Aspergillus niger						N.C.		+++				++
Clonostachys rosea		++	++			N.C.						
Chaetomium nigricolor	+++	+++			++	N.C.	+		+++	++		
Fusarium equiseti						N.C.			++	++		
Fusarium oxysporum	++	+	+		+	N.C.	+	++				
Fusarium verticillioides						N.C.						++
Geotrichum candidum	+++	++	++	+++		N.C.						
Mucor hiemalis	+++	+++	+	+		N.C.			+++		+	++
Penicillium chrysogenum	+	+			+	N.C.	+		+		+	
Trichoderma harzianum	+++	+++		++		N.C.			+	+		++
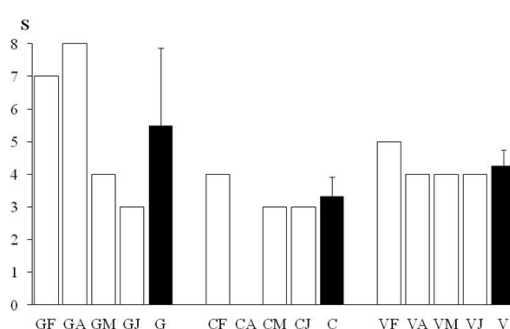
Figure 2: Number of fungal species (S) collected in each stream (G- Gaviola; C-Castellana; V-Vernavola in the four-month survey (F-February; A-April; M-May; J-June). The black columns show the mean number of species (with standard deviation). The CA samples were not collected.

Moreover, according to SIMPER analysis, the most abundant species in the Gaviola Stream was *G. candidum* (47.28% of the total similarity) followed by *Trichoderma harzianum* (19.26% of the total similarity). In the Castellana Stream, the species which contribute most to the similarity between samples were *F. oxysporum* (48.34% of the total similarity) and *C. nigricolor* (25.83%). The community found in the Vernavola Stream was dominated by *Mucor hiemalis* (52.91%) and *C. nigricolor* (26.14%). The dissimilarity between the three fungal communities was statistically confirmed by the ANOSIM test (R=0.518, p=0.002).

Four out of the thirteen isolated fungal taxa showed very low frequencies, less than 5%, and therefore can be considered as occasional: *Acremoniella atra*, *Aspergillus flavus*, *Clonostachys rosea* and *Fusarium verticilloides*. On the other hand, five fungal species have a frequency of about 20%, which can exceed 40% locally: this is the case for *Fusarium oxysporum* and *Geotrichum candidum*. In particular, the latter species was isolated exclusively from the Gaviola Stream, where it was always detected with high abundance and frequency (Table 2, figure 3).
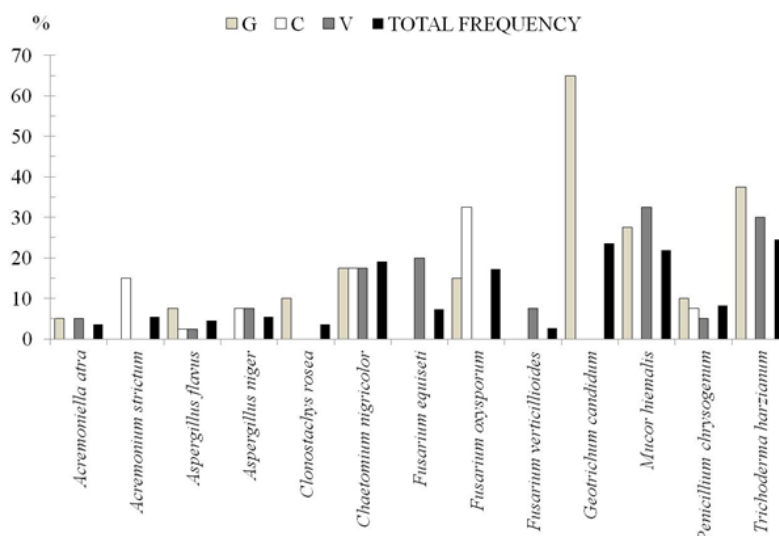
Figure 3: Frequencies of the fungal species detected in each stream. Black columns show the frequency of each fungal species out of the total samples.

G. candidum is a saprophytic fungus of a variety of fruit and vegetables, as well as a component of the microflora of many animals. Nevertheless, its ability to penetrate tropical animals, as an opportunistic agent of disease, has already been reported. For instance, it has been related to lung infection and renal geotrichosis in Galapagos tortoises [19, 20], dermatitis in snakes and pythons [21,22], stomatitis and ophthalmitis in dusky pigmy rattlesnakes [23], and gastrointestinal geotrichosis in adult gorillas [24]. Similar observations have also been documented for bovines (dermatitis in aborted bovine fetus [25]), horses (dermatomycosis [26]), canines (oral ulcers [27,28,29]), disseminated geotrichosis [30] and human beings (disseminated infection in immunosuppressed people [31,32]).

However, no data are available for its hypothetical pathogenicity on strictly aquatic organisms, but it could be considered potentially pathogenic for the Asiatic clam itself as well as for organisms towards which the bivalve can act as a vector.

During our survey in the Gaviola Stream, we noticed abnormal external shell erosion and a reduction of valve thickness in large specimens of *Corbicula fluminea* (Daniele Paganelli, personal observation). Considering that the Asiatic clam population in this stream is intensely infested by *G. candidum*, the hypothesis that this fungal species may affect the bivalves causing an impairment of their shells or preventing them from self-repairing is under evaluation.

Furthermore, some of the other isolated fungi have already been reported to be dangerous for aquatic organisms, e.g. the *phytopathogenic Fusarium* is an agent of diseases for sharks, dolphins, whales, shrimp [33]; the saprotrophic, pathogenic and *mycotoxigenic A.flavus*, frequently associated with sponges [34], *scleractinian corals* [35], soft corals [36] and have also been isolated from diseased tissue of the sea fan *Gorgonia ventalina* in Puerto Rico [37] ;or the species belonging to the wide group of the *Mucoraceae M.hiemalis*, often reported as causal agents of Mucormycosis in fishes and amphibians [38].

Moreover, the following isolated species have also been reported to be able to live in aquatic habitats: the causal agents of opportunistic mycoses in human beings *A.niger*, well-adapted to lakes of the Augustowska Primeval Forest, Poland [39]; *P.chrysogenum*, isolated as endophytic fungus from an unidentified marine red algal species of the genus Laurencia [40] and *T.harzianum*, an opportunistic species found in a large variety of ecosystems including marine habitats and sponge tissues [41].

Finally, it is interesting to make a different consideration regarding *C.nigricolor*, isolated for the first time in India from vegetal detritus, and particularly known for its ability to degrade paper and cellulosic substrates [42]. This species is reported as an unusual etiological agent of human infections (i.e. cutaneous or systemic phaeohyphomycoses), with a high mortality rate among immunocompromised patients [43]. To our knowledge from the literature, so far this species has never been reported in water ecosystems. However, we found *C. nigricolor* in all streams with a medium frequency of about 20%, (figure. 3) and a relatively high abundance (Table. 2). Thus, according to our data, this fungal species constitutes an intimate and stable association with *C.fluminea*.

CONCLUSION

At first sight, the Asiatic clam might seem an unusual fungal niche, and the possibility of an interesting relationship between this bivalve and fungi may sound unlikely. However, it cannot be overlooked that a deep fungal colonization could positively or negatively interfere with (i.e., render a service, compete for foods, modify their lifestyle) every kind of aquatic animals.

The results obtained from this first preliminary mycological investigation support the decision to perform this survey, and they were discussed on the basis of two pieces of evidence that emerged from our study:

1. *C. fluminea* proves to be able to harbour fungi;

2. The role and significance of some of the fungi deserve to be considered and evaluated in more detail.

Regarding the first piece of evidence, the protocol generally recommended for the individuation of fungal endophytes of plants (surface sterilization with sodium hypochlorite, removal of the disinfectant by washing in sterile water, fragmentation into small pieces, deposition on the rich MEA medium) was deliberately applied to the sampled specimens to ensure that the isolated fungi could be considered as intimate colonizers of *C.fluminea*. According to [44], the detected diversity of fungi confirms that, thanks to their small size and their unique adaptability, they can actually enter every kind of natural system and, if appropriate conditions are present, they can permanently occupy it.

Taking into account the second piece of evidence, it emerged that some of the isolated fungi can be potential pathogens for various kinds of animal and thus the presence of *C. fluminea* can also be considered as an ecological problem from this point of view.

To conclude this investigation we can affirm that:

a) There is a characteristic fungal flora associated with the invasive bivalve *C.fluminea*;

b) Some fungal species can be site-specific;

c) Some fungal species can be locally very frequent and abundant;

d) Some fungal species are usually present in all the investigated sites;

e) *Geotrichum candidum*, site-specific in the Gaviola Stream, could be related to pathogenic features for *C.fluminea*;

f) *Chaetomium nigricolor* is reported for the first time in aquatic environments as a permanent resident.

Our study on the fungal species associated with a few Italian samples of *C. fluminea* represents a starting point to extend the knowledge of this community to other sites and to investigate the possible interactions with its host and with other components of the biocoenosis.

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