Project: Molecular screening of the ostracod *Heterocypris incongruens* (Crustacea, Ostracoda) as a pilot project to develop an ecotoxocilogical development kit.

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Reporting period: 1.10.2012-30.3.2013

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Executive summary

Introduction

Ostracods have been used as one of the freshwater invertebrates as model organisms for environmental, paleoenvironmental and evolutionary studies, but also for toxic stress studies and for toxicity monitoring of soil and river sediment (Shuhaimi-Othman et al., 2011). In particular, the freshwater ostracod *Heterocypris incongruens* has been repeatedly used for toxicity test (Chial Belgis at al. 2003, Oleszczuk, 2008, Kuldak at al., 2011, Havel et al., 1995). *Heterocypris incongruens* is a cosmopolitan and common species, inhabiting shallow seasonal (summer) pools and small water bodies (Meisch 2000). *Heterocypris incongruens* is tolerant of different temperatures, salinities variations and can stand low oxygen concentration and organic pollution (Mezquita et al. 1999; Meisch 2000).

Lineages that are at least superficially morphologically identical, but are genetically distinct, are usually misclassified as a single nominal species, while they actually belong to a cryptic species complex (Feckler et al. 2012). Genetic studies have detected cryptic diversity in many animal taxa including ostracods (Crustacea) (e.g. Bode et al. 2010; Schön et al. 2012), with the general implication that there are many more species than were previously recognised (Scheffers et al. 2012). For example, in the ostracod species Eucypris virens, more than 40 genetic species occurring in Europe have been reported (Bode et al. 2010), the highest number for freshwater invertebrate animals. Studies on other crustaceans (Feckler et al. (2012) furthermore revealed that such cryptic, genetic species can differ in their response to ecotoxicological stressors.

Aims

The present study is based on a pilot study of the basin of the River Ledra (NE Italy) monitoring ostracod density, richness and community composition (PROGETTO LEDRA).

The main goals of the present research project are:

a. to test with molecular tools for cryptic diversity in *Heterocypris incongruens* and to determine which phylogenetic (cryptic) species within the species complex would be most suitable for standard application in the laboratory.

b. to develop a molecular phylogenetic framework for assessing the impact of residual of wastewater discharges on ostracod fauna using the said cryptic species of *H. incongruens* as a model.

Results

We screened more than 50 specimens of *H. incongruens* from different geographical areas (Belgium, Germany, Greece, Italy, Marocco, Mongolia, Portugal Spain, Tunisia, Turkey and United Kingdom). Preliminary results showed that:

a. There is an exceptional high genetic diversity among European *H. incongruens*.

b. We can confirm that cryptic, genetic species occur in *H. incongruens*. We found one cryptic *H. incongruens* species which is very widespread in Europe and which could be the ideal candidate for future ecotoxicological tests.

c. The next steps will be to further investigate (cryptic) diversity in *H. incongruens* so that it can be developed as a model tool to investigate the indirect effects of residual and emerging pollutants from wastewater treatment plants on surface waters and other aquatic ecosystems. It can also be a next step to a novel kind of collaboration between environmental engineers and evolutionary ecologists.

Introduction

During the last 20 years, the number of detected cryptic species has exponentially increased (Bickford et al. 2007) due to the availability of molecular (mostly DNA-based) methods for species identification and delimitation (Vogler & Monaghan 2007). The discovery of cryptic lineages throughout all metazoan phyla (Beheregaray & Caccone 2007; Pfenninger & Schwenk 2007) is not only important for fundamental science and taxonomy but has also profound implications for conservation and management (examples in Brown et al. 2007; Elmer et al. 2007; Fontaneto et al. 2008; Gustafsson et al. 2009; Marrone et al. 2010). Indeed, if genetic diversity is cryptic, it is equally difficult to recognize it and to protect it from extinction.

Cryptic diversity is not an isolated phenomenon and inland aquatic crustaceans appear to show high frequencies of cryptic diversity (Murphy et al. 2009; Witt et al. 2006). Also in non-marine ostracods, cryptic species have been reported (Nuanes Brandao et al. 2010; Schön et al. 2012). The most baffling discoveries so far are the close to 40 reproductively isolated lineages ("genetic species") within the single morphological ostracod species, *Eucypris virens* (Bode et al. 2010), in Europe and North Africa, the highest number ever reported from a freshwater invertebrate. In some of the investigated temporary pools, no less than 9 *E. virens* cryptic species occur sympatrically (Bode et al. 2010). The current diversity of non-marine ostracods is about 2,000 morphospecies (Martens at al., 2008). Most morphological ostracod species come from the superfamily Cyprididae. If all cyprid ostracods have as many cryptic species as *E. virens*, current estimates for non-marine ostracods would increase by a factor of 40! Whether cryptic diversity goes together with ecological specialisation or sensitivity, is not yet investigated for non-marine ostracods. However, Feckler et al. (2012) could show that cryptic lineages of the crustacean *Gammarus* have different sensitivities to toxic exposure.

Heterocypris incongruens belongs to the same ostracod superfamily as *E. virens* and It is the most widespread species in Northern Italy where at least 125 different clonal lineages were recognized with allozyme studies and seasonal succession of different ecotypes was described (Rossi et al. 2003; Rossi et al. 2006). Parthenogenetic populations occur nearly world wide and bisexual populations are known from the circum-Mediterranean area, central and eastern Europe. If it shows similar amounts of cryptic diversity, the results of ecotoxicology tests without genetic screening might be doubtful because different cryptic species could differently respond to toxic stress. Thus, it is be a prerequisite for future ecotoxicological experiments to first test ostracods genetically for the possible presence of cryptic diversity and to use only animals belonging to the same cryptic species.

Material and Method

Ostracod samples were collected by sweeping a hand net (mesh size: 250 µm) repeatedly close to the sediment and through the vegetation. All ostracods were fixed in 100% ethanol and kept at 4°C.

Specimens were chosen for analysis at random except that we preferentially used individuals whose valves were open and intact, as those were good indications that specimens were alive and healthy when they were killed.

DNA was extracted from76 ostracods belonging to 29 populations using the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's protocol, after washing each specimen three times with distilled water. In some cases the right valve, containing the most diagnostic features for *H. incongruens*, was retained for

morphometric analysis. Concentrations of extracted DNA were estimated with a nanodrop spectrophotometer. The highest quality DNA eluate was used for PCR amplification of part of the mitochondrial COI gene using specific primers: CRUST CO1 FOR (forward) 5'-CHACRAAYCAYAAAGATATTGG-3'andCRUSTCO1REV1 (reverse) 5'-RAARTARGCTCGDGTRTCTA-3' (see Shearn et al. 2012).
For PCR amplification, the Qiagen Hotstar mastermix was used, with 3-5 µl of DNA template and 0.1 µM of each primer in a total PCR volume of 25 µl. Cycling was performed in a BioRad thermocyclers with an initial denaturation temperature of 94ºC for 50 seconds, followed by 40 cycles with 50 seconds at 72ºC, 50 seconds at 45ºC, 50seconds at 72ºC and a final 10 minute extension step at 72ºC.
Successful PCR products were purified with the GFX™ PCR DNA and gel band purification kit (GE Healthcare) according to the manufacturer's protocol, then sequenced automatically and directly in both directions with the PCR primers and the Big dye kit (ABI) on an ABI 3130X. Sequence chromatograms were viewed in FinchTV (Geospiza Inc., n.d.) and, when present, ambiguities were corrected by manually checking sequence chromatograms. One final consensus sequence per individual was obtained. These sequences were aligned and trimmed to equal length in ClustalX (Larkin et al. 2007), together with the sequences from Van den Broecke (2012) and one outgroup sequence (of the marine ostracod *Macroscapha*).
Phylogenetic reconstruction were conducted in Mega5 (Tamura et al., 2011) using the Kimura 2-parameter (K2P) model, with complete deletion of missing information, with 100 bootstrap replicates. Genetic relationships within and between populations were resolved in parsimonious network constructed at the 95% probability limit using TCS 1.21 (Clement et al. 2000). Unconnected networks were considered as different genetic species as suggested by Hart & Sunday (2007).

Results

We screened *H incongruens* from different geographical areas around the globe (Australia, Belgium, Germany, Greece, Italy, Marocco, Mongolia, Portugal Spain, Tunisia, Turkey and United Kingdom) (Fig. 1 and Tab. 1). Most of the *H. incongruens* investigated here were asexual, except for a sexual population from Italy (Apulia). A total of 70 sequences COI sequences of c.700bp were so far obtained from 29 different *H. incongruens* populations. We also collected *H. reptans* and *H. barbara* to use as comparison in future analysis (Tab.2). The phylogenetic trees show the presence of at least ten European species-like entities plus two entities from Australia and Mongolia, respectively (Fig. 2). In the further description of our results, we will thus focus on these 10 different clades or species-like entities.
Clade A, statistically supported in the phylogenetic tree by a bootstrap value of 75%, was composed by ostracods from all different European countries except Portugal. Specimens from the latter belonged to a separate clade, G.
Clade B, the sister group to Clade A, was formed by only two specimens from Sicily and supported by a bootstrap value of 100%. However, specimens from this Italian region were included also in other two clades (clades D+F).
Clade C joined two ostracods from Belgium together with one from Germany and had a bootstrap support of 100%. It is important to emphasize here that another specimen from the same Belgian locality grouped in a different clade (clade D).

All specimens from Corfù island (Greece) were found together in Clade E with a strong bootstrap value of 100%. On the other hand, there was another specimen from Corfù that belonged to clade A.
All the Portugal ostracods were monophyletic and joined clade G with a high bootstrap value of 93%. There were two well-supported sub-clades which represented the two different Portuguese localities where *H. incongruens* had been sampled (San Marcos and Monte dos Corvos).
Clades H and I were characterised by Australian *Heterocypris species* with a low bootstrap value of 59% and 60%, respectively. With a bootstrap of 98% the well-supported clade L was composed by sexual populations from Apulia (Italy). The specimen from Mongolia (HI3I-coc), located close to the outgroup (the marine ostracod *Macroscapha*) could be not belong to the *incongruens* group species due to the position in the phylogentic tree, faraway from the rest of the *Heterocypris incongruens,* and to the particular valves morphology (Fig. 4).
Heterocypris reptans and *H. barbara* were located in the tree in single branches near the Portugal clades (clade G) and the on formed by samples from Apulia (Clade L) respectively.
The clades identified in the phylogenetic COI tree (see Fig. 2) also formed independent genetic networks (Fig. 3). Twenty different unconnected parsimonious networks plus one network with the outgroup were obtained.
Likewise, all networks also form well-supported clades in the phylogenetic tree. The main clade A contained ten haplotypes from all over Europe (Italy, Germany, Turkey, Greece and the UK). These were all connected with a maximum of 7 mutational steps. *Heterocypris incongruens* from Portugal, however, is an exception to the perfect match between the phylogenetic clades and the networks because there were two independent networks corresponding to the phylogenetic sub-clades with the two different Portuguese localities, San Marcos and Monte dos Corvos.
Another exception was Clade D, being in the phylogenetic COI tree composed of specimens from Belgium, Italy (Sicily) and Turkey (Fig. 2). In the networks, this clade was split into 2 unconnected parsimonious networks (IS4 and H15_coc, in yellow green in Fig.3). The specimens from Sicily (H1I_coc) and Belgium (IS4) had identical COI sequences and built one network with a single haplotype while the one from Turkey (H15_coc) had a different DNA sequence and represents a haplotype in a separate network.
Also Clade L, containing the sexual specimens from Apulia, was split into two haplotypes and independent parsimonious networks (Fig.3). One haplotype was shared between two specimens (F6 and M3) while specimen F2 was separate.

Conclusion

Three remarkable results from these preliminary genetic analyses deserve special attention:

1. There is an exceptional high genetic diversity among European *H. incongruens*. The ostracods from Belgium, Germany, Greece and Italy contain several different genetic entities within the same pond, similarly to what Bode et al. (2010) described from *E. virens*. The high diversity at the DNA level also corresponds with older genetic studies on Italian *H. incongruens* using allozymes (Rossi et al. 2003, 2006).
2. Australian *Heterocypris* species show no genetic similarity to European *H. incongruens* and have thus not been introduced from Europe as *E. virens*

(Koenders et al. 2012) has but form a genuine part of the Australian fauna.

3. There is no clear geographic pattern among the different genetic groups of European *H. incongruens*. The most common Clade A contained ostracods from 10 different European countries, which could indicate that dispersal of ostracods throughout Europe is highly efficient, for example through migrating birds.

To summarize, the molecular dataset being created in this study has enabled us to validate previous taxonomic results indicating a high diversity of *Heterocypris* in Europe (Meisch, 2000; Martens et al., 1998). Additional sampling, PCR amplification and DNA sequencing for another 20 samples has already been conducted and will be incorporated into the phylogenetic framework in the near future. Also, we will apply statistical tests as for example in Martens et al. (2012) to conform that the identified genetic clades represent indeed different cryptic species. All this additional research is crucial for investigating the distributional patterns and the phylogeographic relationships of *H. incongruens* in Europe in more depth.

Additional samples will be collected in the Friuli Venezia Giulia region (NE Italy) and in particular in the Ledra river basin.

The present study will strongly contribute to the knowledge on diversity and phylogeographic structure of the ostracod community in the Ledra river basin (NE Italy).

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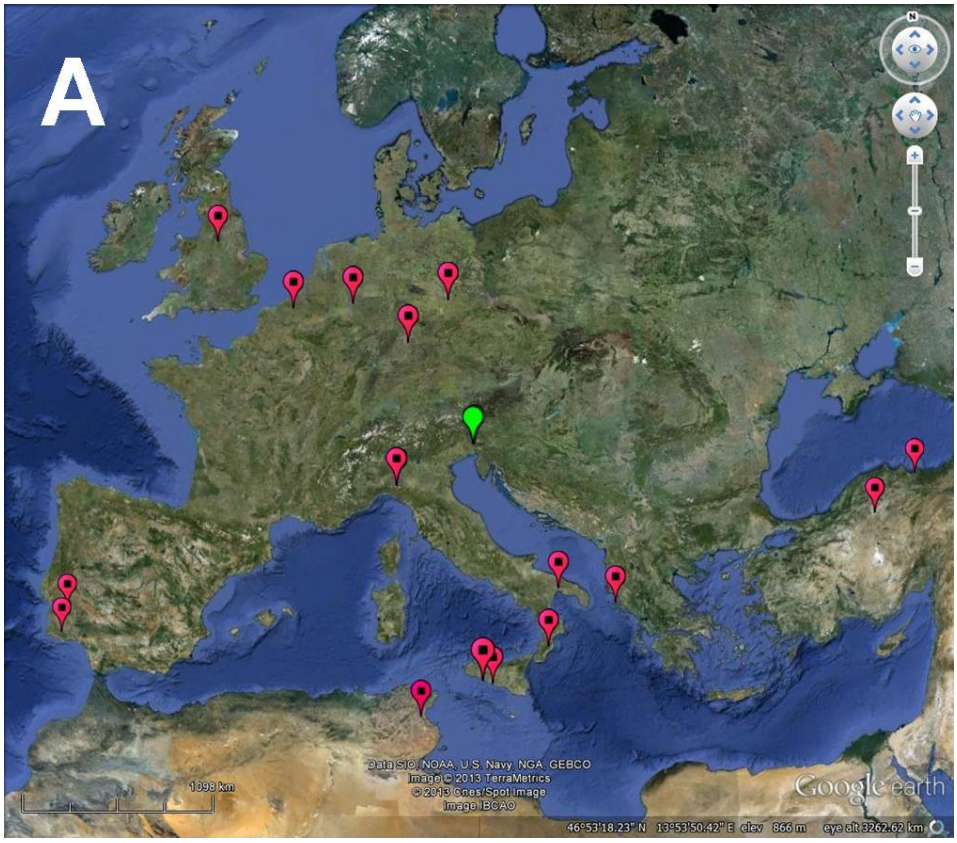


Fig. 1. Map of the sampling sites of *Heterocypris incongruens.* (in pink) and *H. reptans* (in green).

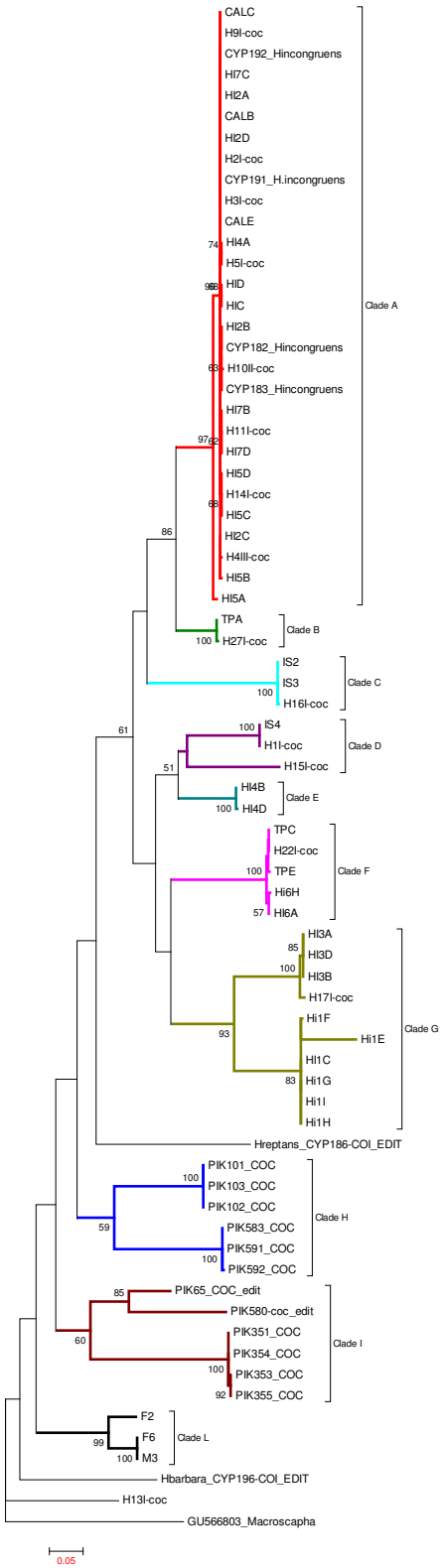


Fig. 2. Phylogenetic tree constructed with novel COI sequences of *Heterocypris incongruens* and published *H. incongruens* sequences. The sequence GU566803 *Macroscapha* represents the outgroup that is required to root the tree. The tree was constructed with Maximum Likelihood methods. Statistical branch support is shown above branches as % of 100 bootstrap replicates. The lengths of the branches is correlated to the genetic distance.

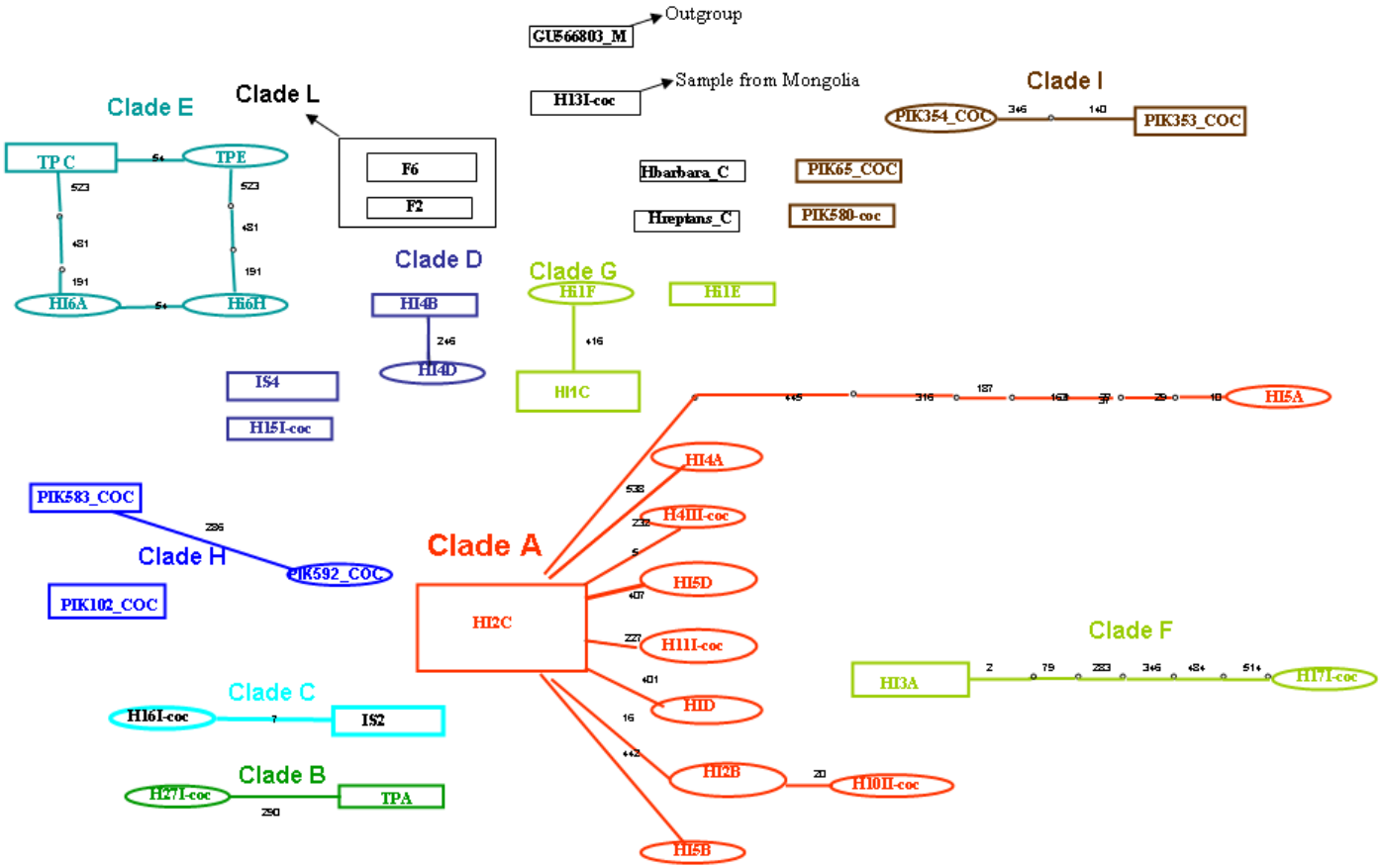
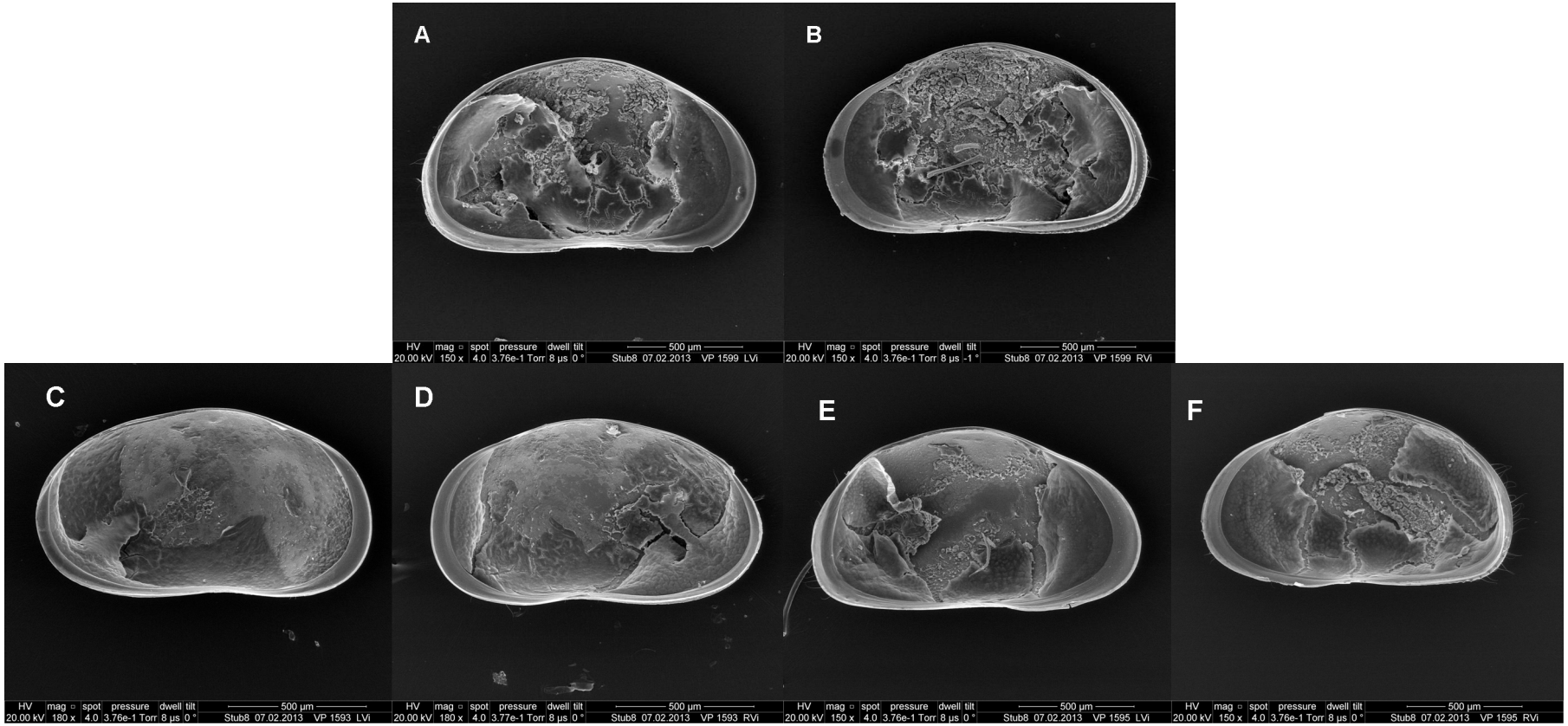


Fig. 3: Parsimonious network constructed at the 95% probability limit with COI sequences from *H. incongruens* specimens. Squares indicate ancestral haplotypes, small circles missing haplotypes.


A
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HV mag spot pressure dwell tilt 500 µm
20.00 kV 150 x 4.0 3.76e-1 Torr 8 µs 0 ° Stub8 07.02.2013 VP 1599 LVi
20.00 kV 150 x 4.0 3.76e-1 Torr 8 µs -1 ° Stub8 07.02.2013 VP 1599 RVi
20.00 kV 180 x 4.0 3.76e-1 Torr 8 µs 0 ° Stub8 07.02.2013 VP 1593 LVi
20.00 kV 180 x 4.0 3.77e-1 Torr 8 µs 0 ° Stub8 07.02.2013 VP 1593 RVi
20.00 kV 150 x 4.0 3.76e-1 Torr 8 µs 0 ° Stub8 07.02.2013 VP 1595 LVi
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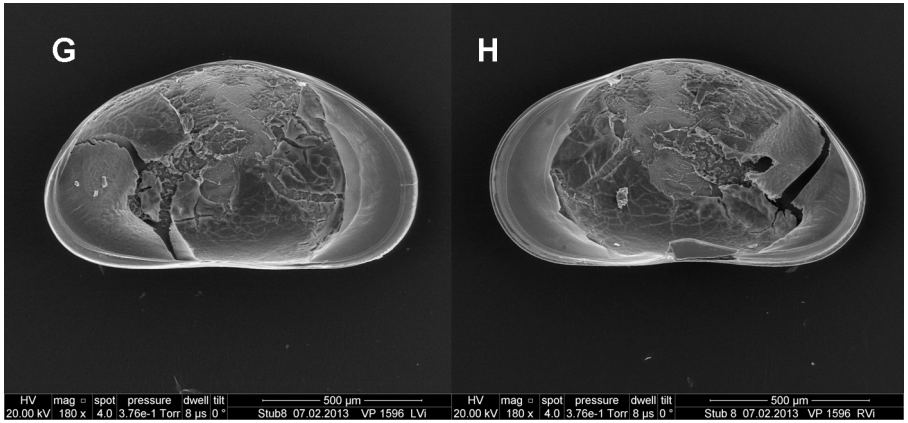

Fig. 4: *Heterocypris* sp. collected in Mongolia (A-B), Italy (C-D), Tunisia (E-F), Italy (G-H). All adult specimens. (**A**): VP1599, female, LV, iv; (**B**): idem, RV, iv; (**C**): VP1593, female, LV, iv; (**D**): idem, RV, iv; (**E**): VP1595, female, LV, iv; (**F**): idem, RV, iv; (**G**): VP1596, female, RV, iv; (**H**): idem, RV, iv. Scale bar: 500 µm.

Table 1: Ostracod samples of *Heterocypris incongruens* from which COI was amplified.

Date	site	N	E	Municipality	Province	Region	Country
04-Mar-07	SXIT099 (Monte Dos Corvos)	37° 25’ 56”	7° 57’ 16”				Portugal
07-Feb-07	SXUK012 (Bamford Weir)	53° 20’ 51”	1° 41’ 51”	Bamford			UK
01-Mar-07	Red Deer Pond	49°49'41"	10°15'59"	Dimbach			Germany
04-Mar-07	SXUK023 (Naunhof Pond, Naunhof highway exit)	51° 18’ 6”	12° 35’ 55”				Germany
16-Jan-06	SXBE005 (Pond with a view)	39°31'41,4"	19°53'59,6"	Komianata	Kornela		Greece
18-Jan-06	SXBE010 (Castaway, Sidari)	39° 47’ 33”	19° 42’ 15”	Perovlades	Corfu		Greece
23-Jan-07	SXBE029 (Maddy shoe ditch)	40°26'13"	33°44'16"	Dedekoy			Turkey
25-Jan-07	SXBE040 (Kitty Pond, Yukiari)	41° 13’ 36”	36° 38’ 15”	Yukiari	Turgutlu		Turkey
26-Jan-07	SXBE041 (Ostracod soup)	40°34'39"	35°41'01"	Amasya/Goküyük			Turkey
04-Jan-06	SXPO008 (N256 2nd pond)	38°27'53"	7°02'00"	Vendinha			Portugal
06-Jan-06	SXPO010 (S. Manços)	38° 26’ 37”	7° 44’ 1”	S. Manços			Portugal
04-Feb-06	SXPO018 (S. Manços)	38°26'37"	7°44'01"	S. Manços			Portugal
04-Mar-07	SX IT095 (N18 1st pond)	38°31'40"	7°48'37"	Evora			Portugal
27-May-05	Pozza c\o albergo Marcesina (Piana di Marcesina)	45°57'47,2"	11°36'20,8"	Asiago	Vicenza	Veneto	Italy
X.2011	CAL049 (Pozza su carrareccia (Pantani Limbi))	4238020	574145	Melicuccà	Reggio Calabria	Calabria	Italy
	TP161				Trapani	Sicilia	Italy
	TF247						Tunisia
21-Aug-12	MNG013	47°04'33"	105°47'57"				Mongolia
12-Aug-12	MNG001	46°49'09"	105°54'49"				Mongolia
16-Aug-12	MNG007	46°59'40"	106°08'08"				Mongolia
18-Nov-12	Fontana 1	44°44'17,09"	9°29'28,81"	Pradovera	Piacenza	Emilia Romagna	Italy
18-Nov-12	Fontana 2	44°44'17,09"	9°29'28,81"	Pradovera	Piacenza	Emilia Romagna	Italy
18-Nov-12	Pozza Acqua Piovana temporanea Cappelletta	44°43'35,81"	9°31'17,60"	Mareto	Piacenza	Emilia Romagna	Italy
25-Dec-12	AG094				Agrigento	Sicilia	Italy
25-Dec-12	AG089				Agrigento	Sicilia	Italy
02-Feb-06	Youssalia (Youssoufia)				Agrigento		Marocco
	Apulia					Puglia	Italy
	Wuppertal						Germany
	Drongen					Drongen	Belgium

Table 2: Ostracod samples of *Heterocypris reptans*.

Date	**site**	**N**	**E**	**Municipality**	**Province**	**Regione**	Country
24-Nov-12	Fontana 1 Cravero	46°08'00,93"	13°33'34,58"	Cravero	Udine	Friuli Venezia Giulia	Italy
24-Nov-12	Fontana 2 Cravero	46°08'00,93"	13°33'34,58"	Cravero	Udine	Friuli Venezia Giulia	Italy
24-Nov-12	Madonnina	46°05'40,27"	13°28'46,76"	Purgessimo	Udine	Friuli Venezia Giulia	Italy
24-Nov-12	Rio lato strada Purgessimo	46°05'38,77"	13°29'05,55"	Purgessimo	Udine	Friuli Venezia Giulia	Italy

Table 3: Ostracod samples of *Heterocypris* sp. from which COI was amplified.

Date	**site**	**N**	**E**	**Municipality**	**Province**	**Region**	**Country**
21-Aug-12	MNG013	47°04'33"	105°47'57"				Mongolia
12-Aug-12	MNG001	46°49'09"	105°54'49"				Mongolia
16-Aug-12	MNG007	46°59'40"	106°08'08"				Mongolia
07-Apr-06	PIK65					Murchinson /Gascoine	Australia
24-Apr-06	PIK101					Pilbara	Australia
24-Apr-06	PIK102					Pilbara	Australia
24-Apr-06	PIK103					Pilbara	Australia
21-Jul-10	PIK351					Kimberley	Australia
21-Jul-10	PIK352					Kimberley	Australia
21-Jul-10	PIK353					Kimberley	Australia
21-Jul-10	PIK354					Kimberley	Australia
05-Jul-11	PIK580					Murchinson/ Gascoine	Australia
05-Jul-11	PIK583					Murchinson/ Gascoine	Australia
05-Jul-11	PIK591					Murchinson/ Gascoine	Australia
05-Jul-11	PIK592					Murchinson/ Gascoine	Australia