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Abstract

Background. During the course of evolution, numerous taxa abandoned canonical sex and reproduced asexually. Examination of the *Cobitis hankugensis* - *Iksookimia longicorpa* asexual complex already revealed important evolutionary discoveries tackling phenomena like interspecific hybridization, non-Mendelian inheritance, polyploidy and asexuality. Yet, as in

other similar cases, the investigation is hampered by the lack of easily accessible molecular tools for efficient differentiation among genomotypes.

Material and methods. Here, we tested the cross-species amplification of 23 microsatellite markers derived from distantly related species and investigated the extent to which such markers can facilitate the genome identification in non-model hybrid complex.

Results. We found that 21 out of 23 microsatellite markers amplified in all genomotypes. Five of them could be used for easy diagnosticity of parental species and their hybrids due to species-specific amplification profiles. We also noted that three markers, i.e. IC654 and IC783 derived from *Cobitis choii* and Iko_TTA01 from *Iksookimia koreensis*, had dosage-sensitive amplification efficiencies of species-specific alleles. This could be further used for reliable differentiation of genome composition in polyploids.

Conclusions. The present study introduces a noninvasive method applicable for diagnosis of ploidy and genome composition of hybrids, which are not clearly distinguished morphologically. We showed that very detailed information may be obtained even from markers developed in distantly related taxa.

Significance statement. Hybridization is being increasingly recognized as a driving force in evolution. Yet, proper detection of hybrids and their ploidy is particularly challenging especially in non-model organisms. Present paper evaluates the power of microsatellite cross-amplification not only in identification of hybrid forms but also in estimating their genome dosage on an example of fish taxon that involves asexuality, hybridization as well as ploidy variation. It thus demonstrates the wide applicability of such cheap and non-invasive tools.

Keywords: microsatellites, cross-amplification, *Iksookimia longicorpa-Cobitis hankugensis* complex, hybridization, asexual reproduction, polyploidy

INTRODUCTION

Although initially neglected in zoological literature, hybridization and polyploidy are attracting considerable research interest as mighty evolutionary mechanisms. Both phenomena are further linked with aberrant reproductive modes leading to the so called asexual lineages with more or less severe deviations from canonical Mendelian reproduction

(Janko et al. 2018). The order Cypriniformes represents diverse group of primarily freshwater fish where incidences of hybridization, polyploidy and asexuality are relatively frequent and new cases are still being discovered in recent time (Li et al. 2014). The group divides into several lineages of which one, the suborder Cyprinoidei, is heavily exploited and explored from both scientific and commercial purposes. Unlike Cyprinoidei, its sister lineage Cobitoidea remains largely understudied although it represents very speciose group. Only recently has the proper taxonomy of this group been investigated, which lead to discoveries of new species (Janko et al. 2005) and even entirely new families (Bohlen and Šlechtová 2009). The group Cobitiodei is adapted to almost every water habitat ranging from standing anoxic waters to high mountain streams and contains several extravagant cases of independently evolved hybrid complexes (including the intergeneric gene exchange (Slechtová et al. 2008)), polyploidizations and asexually reproducing lineages (Kim and Lee 2000, Janko et al. 2007). The reason why this group remains relatively poorly studied lays in its benthic life style promoting conservativeness of body shape and consequent presence of many cryptic species, which may be diagnosed only by molecular markers (e.g. (Janko et al. 2005)). This study focuses on South Korean member of the suborder, the so called *Cobitis hankugensis* - *Iksookimia longicorpa* hybrid complex, which contains two hybridizing species and wide array of asexual diploid and polyploid hybrid lineages, which will also be referred to as genomotypes in the subsequent text. In particular, we describe new molecular tools for proper determination of those forms, which will streamline future research of this model taxon.

The hybridization between *Cobitis hankugensis* and its confamilial relative *Iksookimia longicorpa* was first reported at the Nakdong River tributaries by Kim and Lee (1990) who documented the existence of di- and triploid all-female hybrid population. Later, Lee (1992) reported that ecological and morphological traits of hybrids appear intermediate between their parental species. Based on the results of chromosome analysis, Lee (1992) revealed that hybrids in fact consist of two genomotypes, namely diploid *hankugensis* - *longicorpa* (hereafter HL, where the letter L stands for *longicorpa* genome whereas the letter H stands for *hankugensis* genome) and triploid with two *hankugensis* and one *longicorpa* genomes (hereafter HHL). Additionally, Lee (1995) reported another triploid form with one *hankugensis* and two *longicorpa* genomes (hereafter HLL). Kim and Lee (2000) and Ko (2009) documented an exceptional way of reproduction in these hybrids where diploid females produce unreduced eggs, which accept the sperm of males from one of the parental species and give rise to one or the other type of triploids. Triploid females exclude the entire

genome the parental species contributing the haploid chromosomal set and subsequently upon meiotic divisions of the remaining two chromosomal sets produce haploid egg, which, if fertilized by male of the former species may give rise to diploid clone again. Such diploid and triploid generation alternation has never been described in any other asexual fish to date, making this taxon an outstanding model for the research of aberrant reproductive modes. The summary of known reproductive interactions is provided in Fig. 1.

Although studies of this complex may bring discoveries of general importance, they are complicated by nontrivial morphological identification of the three types of hybrids. Cytological and molecular biological approaches are therefore required for their accurate discrimination. However, while diploid and triploid forms may be easily discriminated through the measurement of erythrocyte cell size or by flow cytometry, the two types of triploids may not be discriminated by the flow cytometry due to the absence of significant differences in DNA content between the parental species. Chromosomal counting thus remains the most reliable differentiation method to date, but it has a fatal disadvantage of being extremely timely and invasive. For these reasons, a new approach is needed for further study of the hybrid complex.

Microsatellite loci analysis, one of the most widely used molecular biology research methods, is an accurate tool for verifying genealogy and identification of relatives, as well as demonstrating genetic diversity by separating and analyzing markers that are inherent in each chromosome (McConnell et al. 1995, Nelson et al. 1998, Smith et al. 1998, Goldstein and Schlrotterer 1999, Beacham et al. 2000, Sunnucks 2000, Tian et al. 2017). To date, the microsatellite markers have been developed for several species of the family Cobitidae, including *C. taenia* (de Gelas et al. 2008), *C. choii* (Bang et al. 2009), *I. koreensis* (Yu et al. 2014), and *Koreocobitis nakdongensis* (Ministry of Environment, South Korea, ME 2011). However, the development of microsatellite markers targeting *C. hankugensis*, *I. longicorpa* and *C. hankugensis* - *I. longicorpa* hybrid has not yet been reported. While the *de novo* development of microsatellite markers is labour and cost intensive (Zane et al. 2002: Squirrell et al. 2003, Thiel et al. 2003, Gonzalez-Martinez et al. 2004, Senan et al. 2014), the cross-amplification using the already identified markers is relatively easily accessible. Therefore, in this study we apply the cross-amplification of markers previously developed microsatellite for related *Cobitis* species on the *C. hankugensis* - *I. longicorpa* hybrid complex with the special aim to discriminate among all hybrid genomotypes including the two types of triploids.

MATERIALS AND METHODS

Sample collection and identification. Sampled fish were treated according to the "Ethical justification for the use and treatment of fishes in research" (Anonymous 2006). This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All surgery was performed under MS-222 anesthesia, and all efforts were made to minimize the pain. The collection of *I. longicorpa*, *C. hankugensis* and their hybrids was carried out in three areas with several localities (Fig. 2, Supplementary Table 1), i.e. in the Seomjin River basin, Imsil, Jeollabuk-do, where *I. longicorpa* occurs, in the Nakdong River basin, Hapcheon, Gyeongsangnam-do, where *C. hankugensis* occurs and in the Nakdong River basin, Namwon, Jeollabuk-do.

The identification of collected specimens was based on previously published methods. In particular, we examined each specimen by morphology, which is known to consistently distinguish both parental species from each other as well as their hybrids (albeit, we stress that morphological analysis may not reliably distinguish among different genomotypes of hybrids) (Kim and Park 2002). The ploidy was evaluated by erythrocyte size measurement (Ko 2009). To cross-validate our determination and to precisely evaluate the genomic composition, we employed karyotype analysis to a subset of specimens. This method provides reliable determination of sexual or hybrid genomotype given that both sexual species differ by chromosomal numbers (*I. longicorpa* 2n = 50; *C. hankugensis* 2n = 48), therefore allowing easy determination of both parental species (hereafter also labeled as LL and HH, respectively) from their diploid (HL, 2n=49) and triploid (HHL 3n = 73 or HLL 3n = 74) hybrid forms (Supplementary Fig. 1).

Finally, to obtain comparative material with known origin and genome composition, we have also performed 4 experimental crosses of parental species to obtain strict F1 HL hybrids, and we also crossed natural diploid HL hybrid females with either LL (3 families) or HH (3 families) males to obtain triploid HHL and HLL hybrids, altogether yielding a total of 133 experimental progeny of verified origin for microsatellite genotyping.

Microsatellite marker selection. For the cross-species amplification analysis, the aforementioned fish samples were scrutinized for previously published microsatellite markers developed for related species of the Cobitidae family. The list of loci is shown in Table 1.

DNA Amplification and Genotyping. For DNA analysis a piece of pectoral fin was dissected from each specimen and stored in 100% Ethyl alcohol. Total DNA was purified with the genomic DNA Prep Kit for blood and tissue (QUIAGEN Co., USA). PCR reactions were completed in a total volume of 50 μℓ, consisting of 2 μℓ of genomic DNA, 1 μℓ of the 10 uM forward (fluorescently labeled) and reverse primer solutions, 24 μℓ of Premix Taq (Takara, Japan) and 22 μℓ of distilled water (Takara, Japan). Polymerase chain reactions for all specimens were executed in GeneAtlas G-02 termocycler (Astec, Japan) with initial denaturing step at 95°C for 5 min and 35 cycles of 30 Sec at 94°C, 30 Sec at 55°C, and 1 min at 72°C. A final extension step at 72°C for 5 min. The PCR amplicons were visualized on a 2% agarose gel stained with LoadingStar (Dyne, Korea) together with negative controls and Takara 1 KB molecular size ladder for preliminary size determination. The final PCR products were run on an ABI-3730XL sequencer (Applied Biosystems, USA) with the size standard at 350 ROX. The resulting electropherograms were analyzed in Peak Scanner v1.0 (Applied Biosystems, USA).

To evaluate whether particular markers bear consistent information about the allelic dosage in diploid and triploid hybrids, we used the Gene scan peak analysis with the Peak Scanner v1.0 (Applied Biosystems, USA) to analyze and compare the relative intensities of alleles in analyzed individuals.

RESULTS AND DISCUSSION

Determination of experimental animals with classical markers. Altogether, based on the classical determination methods including karyotype analysis we selected for marker validation 25 individuals of *I. longicorpa*, 25 of *C. hankugensis*, and also 5 HL, 5 HLL and 5 HHL hybrid individuals. We further included into the analysis 59 natural hybrids (without karyotype analysis) sampled at five sites in the Nakdong River basin, and we also scrutinized 133 progenies generated by artificial crossing experiments with known origin and genomic composition.

Cross-species amplification results and species diagnosticity. The cross-amplification of 23 published markers showed that 19 loci were amplified in all genomotypes of the hybrid complex. Moreover, we further noticed that the IC875 marker did not amplify with *I. longicorpa* but it did amplify in the *C. hankugensis* and hybrids while the Iko_AAT08 marker

did not amplify at *C. hankugensis*, but it did amplify in the *I. longicorpa* and in hybrids (Table 1). We observed that 50.0% of tested loci were monomorphic in *I. longicorpa*, 59.1% in *C. hankugensis*, and 43.5% in all three types of hybrids. The number of alleles per locus, except for monomorphic ones, ranged from 2-6 (mean 3.2) in *I. longicorpa*, 2-9 (mean 4.0) in *C. hankugensis*, 2-5 (mean 2.5) in hybrid (HL type), 2-4 (mean 2.9) in hybrid (HLL type) and 2-5 (mean 2.7) in hybrid (HHL type), respectively.

We note that in each locus, the numbers of detected alleles were always lower than those reported in the reference species for which given microsatellite marker has been developed and where 2-33 alleles per locus/species (mean 12.2) have been reported in the original publications. When compared to the reference species, analyzed hybrids had highest numbers of alleles in markers taken from *C. taenia* (average 2.3 alleles per locus), the second highest numbers of alleles in loci taken from *C. choii* (average 2.2), while the markers taken from different genera showed lowest numbers of alleles, i.e. average 1.5 in *K. naktognensis* markers and 1.4 in *I. koreensis* markers, respectively. This is in line with the general expectation that efficiency of cross-species amplification tends to decrease with increasing phylogenetic distance between the reference species and the target species (Moore et al. 1991, Peakall et al. 1998). This study used the cross-amplification between species of the same genus or family and showed relatively high amplification efficiency. However, nearly half of essayed loci appeared as monomorphic and many of other loci shared the same alleles between species, making them of limited use for species diagnosis. Future studies of genetic diversity of *I. longicorpa* and *C. hankugensis* and their hybrids would certainly profit from the direct development of microsatellite loci from their own DNA.

Nevertheless, we discovered three loci, which seem very useful for fast and efficient identification of genomotypes from the studied hybrid complex because they possess non-overlapping allelic size ranges between species. Specifically, the loci IC654, IC783 and Iko_TTA01 always distinguished between the specimens identified as pure *I. longicorpa* and *C. hankugensis*, respectively, while they always provided amplification products of both species in the hybrid individuals. Furthermore, we found two additional loci with selective amplification (IC875, Iko_AAT08), where one sexual species was characterized by absence of amplification, while the other species and all hybrid individuals provided specific amplification product (Table 1).

This altogether suggests that tested cross-amplification identified three markers with species-specific allelic variants and two loci with species-selective amplification that may be

used as haploid detection markers for *C. hankugensis* and *I. longicorpa*, respectively. In addition, some other loci also appear as useful for subsequent population genetic studies given they possess moderate number of alleles per species, which may allow for frequency-based analyses.

Hybrid detection and allele dosage effects. Given that the scope of this paper was to find fast and efficient method to discern parental species and hybrid genomotypes, we will describe in the following text the properties of three markers that we propose for such a purpose given their ability to diagnose both species as well as the ploidy of hybrid individuals. The IC654 and IC783 markers derived from *C. choii* and the Iko_TTA01 marker derived from *I. koreensis* were of particular interest for us as they were fixed for different alleles in both parental species and showed consistent presence of both species-specific amplification products in hybrids with different relative peak intensities depending on the genotypes (Fig. 3).

The patterns were straightforward in IC654 and Iko_TTA01 markers (Fig. 3, A-B) since in diploid hybrid HL genomotype with one genome of *I. longicorpa* and the other of *C. hankugensis*, the heights of the amplified peaks were similar to each other. In triploid hybrid HLL genomotype with two genomes of *I. longicorpa* and one of *C. hankugensis*, the allele peak intensity of *I. longicorpa* – specific allele was approximately twice as strong as that of *C. hankugensis* – specific allele, while in triploid hybrid HHL genomotype the situation was opposite with approximately double intensity of the *C. hankugensis* allele compared to *I. longicorpa* allele. The patterns in IC783 were more complicated by the presence of two alleles in *I. longicorpa*. Consequently, the diploid HL form always possessed one allele diagnostic for *I. longicorpa* and the other for *C. hankugensis*, HHL triploids also possessed two alleles with clear dosage pattern, but HLL triploids either possessed two alleles with the apparent dosage pattern, or three alleles, each with similar intensity (Fig. 3, C).

To verify the possibility of applying the three selected markers (IC783, IC654 and Iko_TTA01) for identification of unknown hybrid genomotype, we demonstrate the relative size ratio of the minor peak to the major one (Fig. 3, D-F). As a result, three hybrid genomotypes were clearly distinguished by the ratio of the peaks. Diploid HL genomotype had relatively similar intensities of the less intense allele, i.e. 92.7% (IC654), 84.4% (IC783) and 87.9% (Iko_TTA01), while in triploid HLL genomotype, the ratios were 54.3%, 62.3% and 45.3%, respectively, and in HHL genomotype, we found 51.6%, 45.6% and 54.1%, respectively (Fig. 3, D-F). Finally, we also plotted the average relative values of the total of

three markers for each genomotype, (Fig. 3, G), where on average the value in HL type was 89.8%, in HLL type was 55.3%, HHL type was 49.2%. This result strongly supported that the microsatellite marker can be used to the correct method of discrimination of known genomotypes of the *C. hankugensis* - *I. longicorpa* complex.

To date, the identification of *C. hankugensis* - *I. longicorpa* hybrid complex had a fatal disadvantage that it requires complex processing and fish sacrifications. In this study, we provided reliable identification method of the *C. hankugensis* - *I. longicorpa* hybrid complex using microsatellite markers through a single Genescan analysis using only a small piece of fin tissues. This has the great advantage that the fish are kept alive and can be used for additional hybridization experiments by reducing the stress.

Microsatellite markers have indeed been previously used to identify hybrid groups of fish, including the family Cobitidae, (e.g. You et al. 2007 de Gelas et al. 2008) and these markers have also been applied to polyploid hybrids, (e.g. Janko et al. 2012 Mishina et al. 2014), when triploids were typically inferred by possession of three peaks in at least one locus. However, the identification method proposed in this study suggests that microsatellite markers can be used as a powerful method to determine triploids even in cases when hybrids possess no more than two alleles, relying on the relative amplification intensities of species-specific alleles.

In a summary, the cross-species amplification of microsatellite markers can be used as easy and fast identification method in studies of reproductive modes of investigated hybrids.

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Fig. 1. The summary of known reproductive interactions between parental species and hybrid forms of the *Cobitis hankugensis* - *Iksookimia longicorpa* hybrid complex

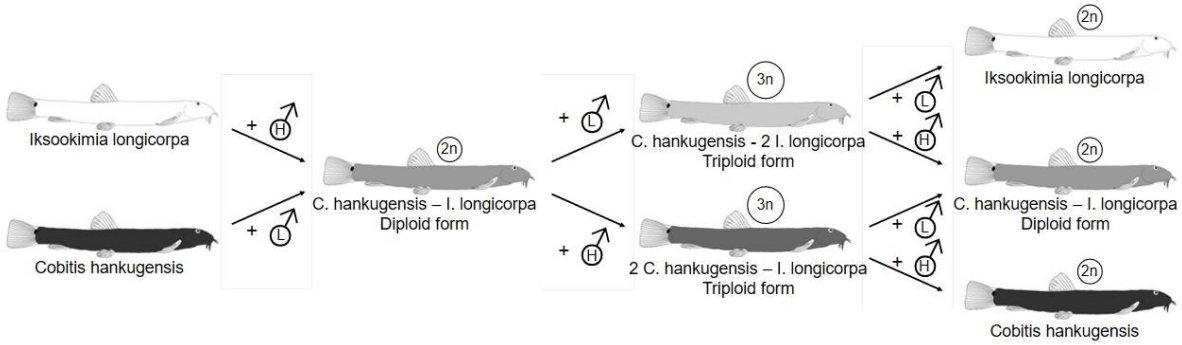


Fig. 2. Sampling localities of *Cobitis hankugensis* - *Iksookimia longicorpa* hybrid complex used in this study

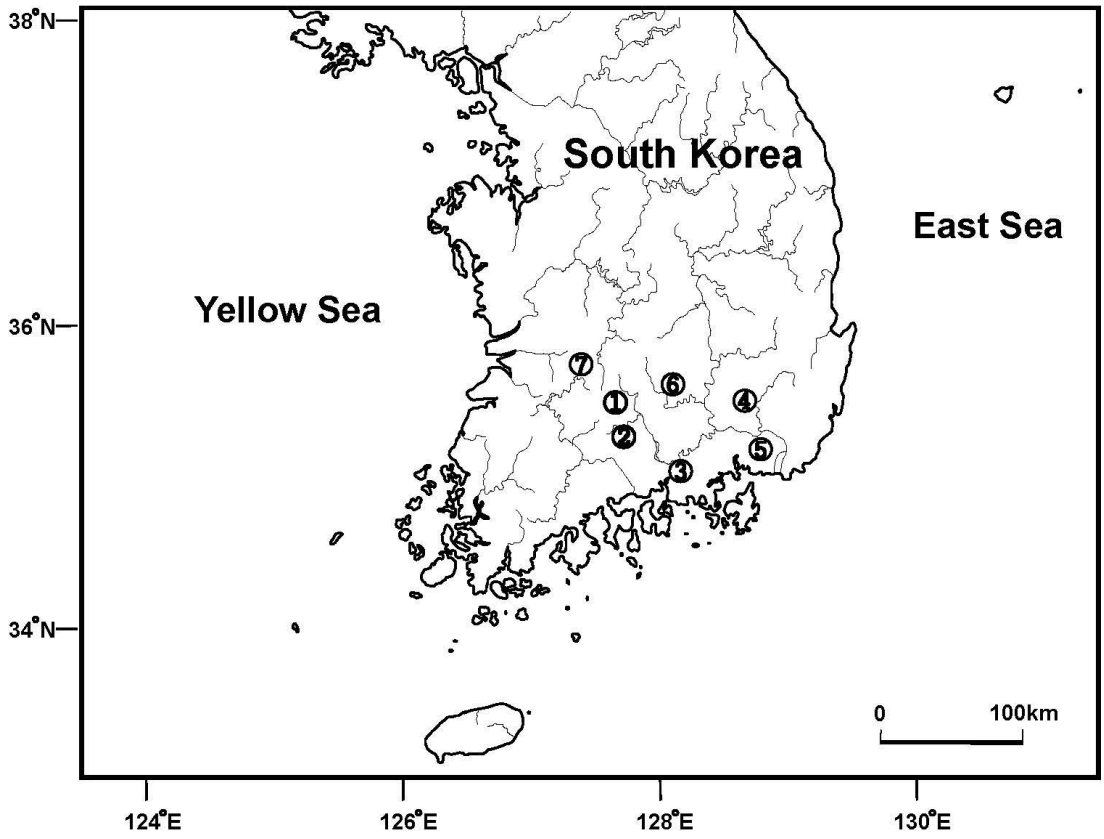
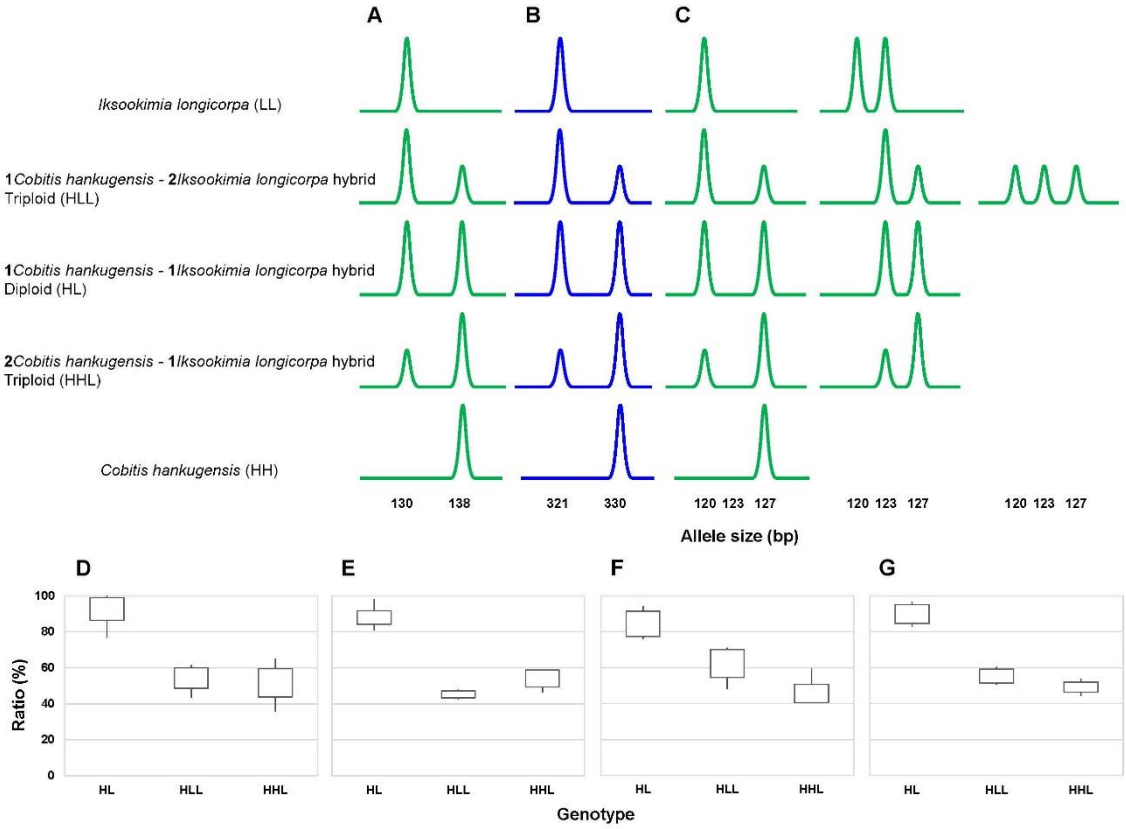


Fig. 3. Demonstration of Microsatellite analysis of IC654 (A), Iko_TTA01 (B) and IC783 (C) loci in *Cobitis hankugensis* - *Iksookimia longicorpa* hybrid complex. Upper panels A-C show the electropherograms of the three loci in all biotypes. Boxplots in the lower panel

depict for each locus the relative intensity (%) of the minor peak to the major one (D-F) and the average relative values of the total of three markers for each genomotype (G)



Supplementary Fig. 1. Metaphase plates and karyotypes of two triploid *Cobitis hankugensis* - *Iksookimia longicorpa* hybrids. A: genotype HLL; B: genotype HHL

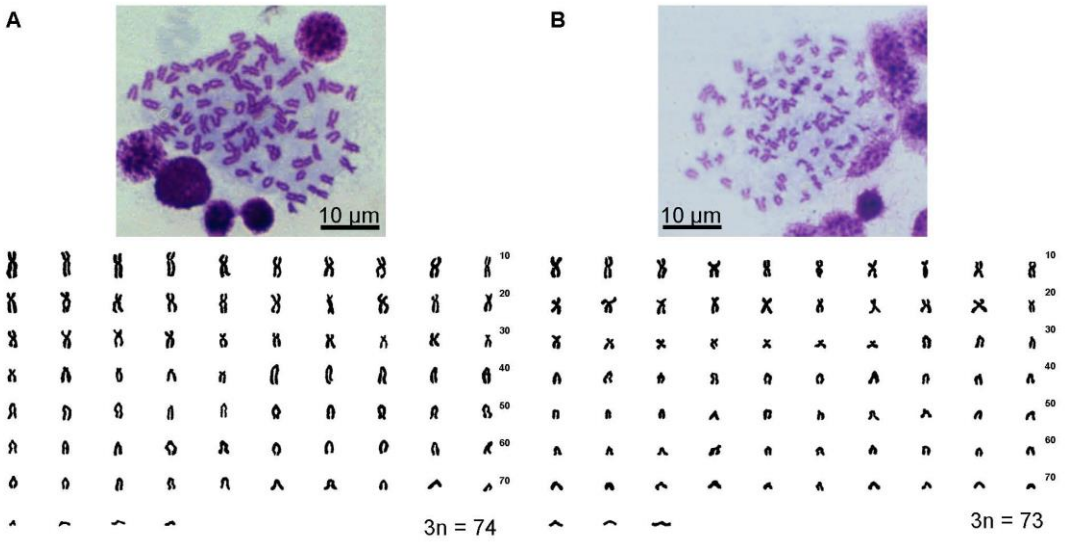


Table 1

Details of 23 microsatellite markers used for *Cobitis hankugensis* - *Iksookimia longicorpa* hybrid detection. Cross-amplification results are indicated for both *Iksookimia longicorpa*, *Cobitis hankugensis* species. Five markers with suited properties for genomotype identification and dosage effects are highlighted in bold

Locus	Primer sequence (5' → 3')		Repeat motif	Reference species	Accession No.	Reference species		*Iksookimia longicorpa*		*Cobitis hankugensis*	
						N_A	allele size (bp)	N_A*	allele size (bp)	N_A	allele size (bp)
Cota_006	F:	HEX-GCAGGTACAGAACCCCGACATGG	TTG/CTAT	*Cobitis taenia*	EU276579	11	336-374	2	163-165	2	163-165
	R:	AGTACGGCCCTATGGGGTTTGAC		(de Gelas et al., 2008)							
Cota_025	F:	6-FAM-TGCGTTTACAAGATTCGTGTGGAC	CACG		EU276580	3	144-160	2	42-52	2	42-52
	R:	GCTGCATATGAGTAACATGTCTG									
Cota_032	F:	6-FAM-TGGTCATGACTGGCACACCGTC	TCTT		EU276582	2	232-236	2	271-290	4	271-301
	R:	AGGAGGTTTGAAGAAGGGCAAG									
Cota_033	F:	HEX-TTTCTGAATCAAGAGCCCAGCAGT	AGAC		EU276583	3	211-235	2	203-207	1	207
	R:	AGATATGACATCCAATCACACGCT									
Cota_037	F:	6-FAM-GCACTCGAGTCGATTCGGTGGCGC	GA		EU276584	3	272-276	6	275-304	4	280-298
	R:	GTAATCAATCAGTCCAAAGCACTT									
Cota_093	F:	6-FAM-CCCTGGGAGTTCTCAGCAGGACTG	AC		EU276586	4	341-357	1	304	1	304
	R:	ATAATGCACATTGTTGGGCTGC									
IC248	F:	HEX-CACTCTGAGGCGAAACTGGAG	CA	***Cobitis choii***	EU252088	24	123-187	6	117-148	4	107-119
	R:	TCAAATCATATAGTGCAGCCAAGC		**(Bang et al., 2009)**							
IC252	F:	HEX-AATGAGACGGGTAACTTGTGTATG	CA		EU252089	12	188-218	1	155	1	155
	R:	GCTGATCTATGATTGGTTGTGTC									
IC276	F:	6-FAM-GTAACTCCGGGCGTGTAACTCTG	GT		EU252090	14	82-114	1	70	1	70
	R:	CACTGTAGAACCCAGCCAAAACC									
IC372	F:	6-FAM-ACACGCACACCTATTACAACCTA	AC		EU252091	33	77-169	2	86-90	2	86-90

	R:	GATTTGCCAGTGTGCTAATTG									
IC434	F:	6-FAM-TCCACCATGACCATTTTTACATA	AC		EU252092	23	83-165	1	78	1	78
	R:	GGTGTCTGGATCTCATCTTGAA									
IC645	F:	6-FAM-CTCTGAGACAACTCGGTAGTCCC	CA		EU252095	19	161-225	1	189	1	189
	R:	CACATACATGGCCTGCAACAT									
IC654	**F:**	**HEX-TGAGCCGACACTAGAAACAGAGC**	**CA**		**EU252096**	**14**	**158-208**	**1**	**130**	**1**	**138**
	R:	**GACAAAGTGCAGGCACAGAATG**									
IC783	**F:**	**HEX-GGAGAAGATGTGATGGAGATG**	**AC**		**EU252098**	**22**	**146-196**	**2**	**120-123**	**1**	**127**
	R:	**ATATTATGATGGGAAGACACGAC**									
IC839	F:	6-FAM-TTGTTCCCCTCTGAAACCCAATC	CA		EU252100	13	99-125	5	92-110	5	82-94
	R:	GTGTTAGCCCGTGTGCCAAAG									
IC875	**F:**	**HEX-AGCGGTGTGGATGTGAATGCTAA**	**CA**		**EU252101**	**22**	**132-182**		**-**	**9**	**134-158**
	R:	**CTTGTCAGGCTCTGGCACTCG**									
Iko_AAT08	**F:**	**6-FAM-GTGATGCAAATGTCTTCTGTGT**	**ATT**	***Iksookimia koreensis***	**KJ588473**	**5**	**147-163**	**2**	**125-135**		**-**
	R:	**CAAATCTTTCCTTTGTCTTTGG**		**(Yu et al., 2014)**							
Iko_TTA01	**F:**	**6-FAM-ACATTAGTGGGGTAAGATGTGC**	**TTA**		**KJ588474**	**8**	**180-238**	**1**	**321**	**1**	**330**
	R:	**AAGGAAGGAATAGGGTAAGCTG**									
KN03	F:	HEX-TTTGAGAATTGACAAAATCACTGC	CA	*Koreocobitis naktongenesis*	JN203057	8	134-156	1	116	1	116
	R:	TGATATCATCGGTGTAAATGTTTAAGA		(ME, 2011)							
KN16	F:	HEX-CGACGTAGAGTCAAAAGTGCG	CA		JN203058	10	135-157	1	126	1	126
	R:	TGGAGATCAGGTTACGGGTG									
KN20	F:	HEX-TTGTGCTGATAACACATCCTGC	CA		JN203059	10	144-172	1	137	1	137
	R:	GATTGAATCATCCGCAGAGC									
KN25	F:	6-FAM-CGTTCCCCTCAGGTCTCAAT	CA		JN203060	9	275-295	4	293-313	4	307-313
	R:	CCTGCAGTTTTCAGCCAAGA									
KN34	F:	6-FAM-CCAGTGGACATCTGCAACAAC	TG		JN203062	9	273-289	1	286	1	286
	R:	GCCCTGCTAGTGAGGAACAA									

* N_A = number of alleles

Supplementary Table 1

Study localities of *Cobitis hankugensis* - *Iksookimia longicorpa* hybrid complex used in this study

No.	River basin	Locality	GPS
1	Ram stream	Inwol-myeon, Namwon-si, Jeollabuk-do	35°27'27.2"N; 127°36'25.6"E
2	Nam river	Saengcho-myeon, Sancheong-gun, Gyeongsangnam-do	35°28'46.9"N; 127°50'56.9"E
3	Banseong Stream	Ibanseong-myeon, Jinju-si, Gyeongsangnam-do	35° 9'51.8"N; 128°17'44.8"E
4	Cheongdo Stream	Punggak-myeon, Cheongdo-gun, Gyeongsangbuk-do	35°38'37.2"N; 128°37'25.8"E
5	Unjeong stream	Muan-myeon, Miryang-si, Gyeongsangnam-do	35°29'37.6"N; 128°40'11.1"E
6	Hwanggye stream	Yongju-myeon, Hapcheon-gun, Gyeongsangnam-do	35°30'20.3"N; 128° 6'17.2"E
7	Oknyeodong stream	Unam-myeon, Imsil-gun, Jeollabuk-do	35°39'35.3"N; 127° 9'20.0"E