Cross-species amplification of microsatellites and identification of polyploid hybrids by allele dosage effects in *Cobitis hankugensis* × *Iksookimia longicorpa* hybrid complex (Actinopterygii: Cypriniformes: Cobitidae)

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Abstract

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. 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 the non-model hybrid complex. We found that 21 out of 23 microsatellite markers were amplified in all genomotypes. Five of them could be used for easy diagnostics 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* Kim et Son, 1984 and Iko_TTA01 from *Iksookimia koreensis* (Kim, 1975), had dosage-sensitive amplification efficiencies of species-specific alleles. This could be further used for reliable differentiation of genome composition in polyploids. The presently reported study introduces a noninvasive method applicable for the 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. 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. The present paper evaluates the power of microsatellite cross-amplification not only in the identification of hybrid forms but also in estimating their genome dosage on an example of a 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 a 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 times (Li et al. 2014). The group divides into several lineages of which one, the suborder Cyprinoidae, is heavily exploited and explored for both scientific and commercial purposes. Unlike Cyprinoidae, its sister lineage Cobitoidea remains largely understudied although it represents a 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 Cobitoidea 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 (Šlechtová 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 lifestyle promoting conservativeness of body shape and the consequent presence of many cryptic species, which may be diagnosed only by molecular markers (e.g., Janko et al. 2005). This study focuses on a South Korean member of the suborder, the so-called Cobitis hankugensis × Iksookimia longicorpa hybrid complex, which contains two hybridizing species and a wide array of asexual diploid and polyploid hybrid lineages, which will also be referred to as genotypes in the subsequent text. In particular, we describe new molecular tools for the 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 (unpublished thesis) reported that ecological and morphological traits of hybrids appear intermediate between their parental species. Based on the results of chromosome analysis, Lee (unpublished thesis) revealed that hybrids consist of two genotypes, namely diploid hankugensis × longicorpa (hereafter H and L, where the letter L stands for longicorpa genome whereas the letter H stands for hankugenisis 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 of the parental species contributing to the haploid chromosomal set and subsequently upon meiotic divisions of the remaining two chromosomal sets produce a haploid egg, which, if fertilized by a 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 non-trivial 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.

**Figure 1.** The summary of known reproductive interactions between parental species and hybrid forms of the Cobitis hankugensis × Iksookimia longicorpa 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 Schlötterer 1999; Bea cham 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 Cobitis taenia Linnaeus, 1758 (see de Gelas et al. 2008), several species of the family Cobitidae, including date, the microsatellite markers have been developed for (see Smith et al. 1998; Goldstein and Schlötterer 1999; Bea-

chromosome (McConnell et al. 1995; Nelson et al. 1998; arating and analyzing markers that are inherent in each ars), to cross-validate our determination and to pre-
dify was evaluated by erythrocyte size measurement (Ko-

type analysis to a subset of specimens. This method cely evaluate the genomic composition, we employed -

togenesis, I. longicorpa, and C. hankugensis × I. longicor-

pa hybrid has not yet been reported. While the de novo
development of microsatellite markers targeting C. hanku-
gensis, I. longicorpa, and C. hankugensis × I. longicor-

pa provides reliable determination of sexual or hybrid ge-

nomotype given that both sexual species differ by chro-

mosomal numbers (I. longicorpa 2n = 50; C. hankugensis 2n = 48), therefore allowing easy determination of both parental species (hereafter also labeled as LL and HH, re-

spectively) from their diploid (HL, 2n = 49) and triploid (HHL 3n = 73 or HLL 3n = 74) hybrid forms (Fig. 3).

Finally, to obtain comparative material with known or-

igin 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 fam-

ilies) males to obtain triploid HHL and HLL hybrids, al-

together yielding a total of 133 experimental progeny of verified origin for microsatellite genotyping.

**Materials and methods**

**Sample collection and identification**

Sampled fish were treated according to the “Ethical jus-
tification 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 In-
stitutes of Health (Anonymous 2011b). The fish dissection was performed under MS-222 anesthesia, and all efforts were made to minimize the pain. The collection of I. lon-

gicorpa, C. hankugensis, and their hybrids was carried out in three areas with several localities (Fig. 2, Table 2), i.e., in the Seomjin River basin, Imsil, Jeollabuk-do, where I. longicorpa occurs, in the Nakdong River basin, Hap-

cheon, 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 consist-
tently distinguish both parental species from each other as well as their hybrids (albeit, we stress that morphological analysis may not reliably distinguish among different ge-

nomotypes of hybrids) (Kim and Park 2002). The ploidy was evaluated by erythrocyte size measurement (Ko 2009). To cross-validate our determination and to pre-
cisely evaluate the genomic composition, we employed karyotype analysis to a subset of specimens. This method

**Microsatellite marker selection**

For the cross-species amplification analysis, the afore-

mentioned 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 (QIAGEN 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 GeneArtas G-02 thermocy-

cler (Astec, Japan) with the initial denaturing step at 95°C for 5 min and 35 cycles of 30 s at 94°C, 30 s at 55°C, and

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

**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 genotyping type identification and dosage effects are highlighted in bold.

| Locus | Primer sequence (5′ → 3′) | Repeat motif | Reference species | Accession No. | Reference size [bp] | Iksokimia longicorpa | Cobitis hankugensis | N* A Allele size [bp] | N* A Allele size [bp] |
|-------|--------------------------|--------------|-------------------|---------------|---------------------|---------------------|---------------------|-------------------|-------------------|
| Cota_006 | F: HEX-GCAGTGACAAACCCCGACTGAG | TGG/CTAT | Cobitis taenia (see de Gelas et al. 2008) | EU276579 | 336–374 | 2 | 2 | 163–165 | 2 | 163–165 |
| Cota_025 | F: 6-FAM-TGCGTTTACAAGATTCGTGTGAC | CAGG | | EU276580 | 144–160 | 2 | 42–52 | 2 | 42–52 |
| Cota_032 | F: 6-FAM-TGTCGATGCGACCCACTGTC | TCTT | | EU276582 | 232–236 | 2 | 271–290 | 4 | 271–301 |
| Cota_033 | F: HEX-TTTCGAATCAAGACGCCCAGCTG | AGAC | | EU276583 | 211–235 | 2 | 203–207 | 1 | 207 |
| Cota_037 | F: 6-FAM-GACTTGAGCTGGTCGTCGCGGCG | GA | | EU276584 | 272–276 | 6 | 275–304 | 4 | 280–298 |
| Cota_093 | F: 6-FAM-CCTGGGAGTTCTCAGGACTG | AC | | EU276586 | 341–357 | 1 | 304 | 1 | 304 |
| IC248 | F: 6-FAM-TGCGTTTACAAGATTCGTGTGAC | CAGG | | EU252088 | 123–187 | 6 | 117–148 | 4 | 107–119 |
| IC252 | F: HEX-AATGAGACGGGTAACTTGTAT | | | EU252089 | 128–218 | 1 | 155 | 1 | 155 |
| IC276 | F: 6-FAM-TGAACTCGGCGGCTAATCTG | GT | | EU252090 | 82–114 | 1 | 70 | 1 | 70 |
| IC372 | F: 6-FAM-ACCGCACACTATACCATTAACAG | AC | | EU252091 | 77–169 | 2 | 86–90 | 2 | 86–90 |
| IC343 | F: 6-FAM-TTCACCATAGACAAAAAAAAA | AC | | EU252092 | 83–165 | 1 | 78 | 1 | 78 |
| IC645 | F: 6-FAM-TCTGAGACAACTCTGGTATCC | CA | | EU252095 | 161–225 | 1 | 189 | 1 | 189 |
| IC654 | F: HEX-TGACGGGACTGATGACACAGACGC | CA | | EU252096 | 148–208 | 1 | 130 | 1 | 138 |
| IC783 | F: HEX-GGAGAAGATGATTGGAGATGAG | ATATTAGTGGGAAAGACAGAC | | EU252098 | 146–196 | 2 | 120–123 | 1 | 127 |
| IC859 | F: 6-FAM-TGTGTTCCCTCTGGAAACCCATC | CA | | EU252100 | 119–160 | 5 | 92–110 | 5 | 82–94 |
| IC875 | F: 6-FAM-AGGGGTTTGGATGGAAGTTGCTA | CA | | EU252101 | 122–182 | 2 | 9 | 134–158 |
| Iko_AAT08 | F: 6-FAM-TGATGACAAATGCTTCCTTCTG | ATT | Iksookimia koreensis (see Yu et al. 2014) | KJ588473 | 147–163 | 2 | 125–135 | – | – |
| Iko_TTA01 | F: 6-FAM-ACATTAGTGGGGTAAATGTCG | TTA | | KJ588474 | 180–238 | 1 | 321 | 1 | 330 |
| KN03 | F: HEX-TTTGAGATGACTGACAAACACTGTC | CA | Koreocobitis naktoon-genesis (see Anonymous 2011) | JN203657 | 134–156 | 1 | 116 | 1 | 116 |
| KN16 | F: HEX-CACTGACAGTAACACCTCTG | GCACAGAC | | JN203658 | 105–137 | 1 | 126 | 1 | 126 |
| KN20 | F: 6-FAM-TGATGACAAATGCTTCCTTCTG | CA | | JN203659 | 140–172 | 1 | 137 | 1 | 137 |
| KN25 | F: 6-FAM-CCAGGCTGATGGCAGCTA | CA | | JN203660 | 275–297 | 4 | 307–313 | 4 | 307–313 |
| KN34 | F: 6-FAM-CCAGGCTGATGGCAGCTACACAC | TG | | JN203662 | 273–289 | 1 | 286 | 1 | 286 |

* N* A = number of alleles.

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).

**Results and discussion**

**Determination of experimental animals with classical markers**

Altogether, based on the classical determination methods including karyotype analysis we selected for marker vali-
morphic ones, ranged from 2–6 (mean 3.2) in hybrids. The number of alleles per locus, except for mono-(mean 2.7) in hybrid (HHL type), 2–4 (mean 2.9) in hybrid (HLL type), and 2–5 (mean 4.0) in hybrid (HHL type), respectively.

Nakdong River basin, and we also scrutinized 133 progenies (without karyotype analysis) sampled at five sites in the basin. We further included into the analysis 59 natural hybrids of the same genus or family and showed relatively high genetic diversity of C. hankugensis.

Cross-species amplification results and species diagnostics

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 per species (mean 12.2) have been reported in the original publications. When compared to the reference species, analyzed hybrids had the highest numbers of alleles in markers taken from C. taenia (mean value 2.3 alleles per locus), the second-highest numbers of alleles in loci taken from C. choii (mean value 2.2), while the markers taken from different genera showed lowest numbers of alleles, i.e., mean value 1.5 in K. naktongensis markers and 1.4 in I. koreensis markers, respectively. This is in line with the general expectation that the 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 assayed 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 the genetic diversity of I. longicorpa and C. hankugensis and their hybrids would certainly profit from the direct development of microsatellite loci from their 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 a 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 a 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 the consistent presence of both species-specific amplification products in hybrids with different relative peak intensities depending on the genomotypes (Fig. 3).

The patterns were straightforward in IC654 and Iko_TTA01 markers (Fig. 4A–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*—

![Figure 4. 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 mean relative values of the total of three markers for each genomotype (G).](image-url)
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. 4C).

To verify the possibility of applying the three selected markers (IC783, IC654, and Iko_TTA01) for the identification of unknown hybrid genomotype, we demonstrate the relative size ratio of the minor peak to the major one (Fig. 4 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. 4, D–F). Finally, we also plotted the mean relative values of the total of three markers for each genomotype, (Fig. 4, G), where the mean 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 in that it requires complex processing and fish sacrifice. In this study, we provided a 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 the 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 an easy and fast identification method in studies of reproductive modes of investigated hybrids.

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