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RESEARCH ARTICLE

The genetic approach for assessing sea trout stock enhancement efficiency – An example from the Vistula River

Anna Wąs-Barcz[1], Rafał Bernaś[1], Roman Wenne

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Abstract. Many countries in the Baltic Sea basin have initiated enhancement programs for Baltic migratory sea trout, *Salmo trutta* L., to compensate for losses stemming from anthropogenic pressure that has resulted in the declining population abundance of this species. Regular stock enhancement has been conducted in Poland since the 1960s. Currently, over one million sea trout smolts are released into Polish rivers annually. In most Baltic countries, including Poland, stock enhancement depends on hatcheries producing material using spawners caught in native rivers. However, increasing difficulty obtaining spawners in recent years in Poland has meant that stock enhancement performed in the Vistula has been done largely with material obtained from broodstocks. Simultaneously, there is a lack of information regarding the proportion of wild and cultured sea trout in this river basin. This paper is a review of methods applied to identify individuals from natural and artificial sea trout spawning in rivers, and it proposes using genetic techniques as an alternative to traditional marking methods. A set of 13 microsatellite loci are proposed that are characterized by high selectivity. Using negative controls while simulating the assignment of parental pairs revealed that the number of loci in the set was highly significant and should not be reduced. This method could be useful in the proposed assessment of the proportions of wild and cultured fish in Polish rivers.

Keywords: sea trout, microsatellite analysis, individual identification, stocking efficiency, assessment, enhancement programs

[1] The authors contributed equally to this work.

A. Wąs-Barcz [✉]
National Marine Fisheries Research Institute
Kołłątaja 1, 81-332 Gdynia, Poland
e-mail: awas@mir.gdynia.pl

R. Bernaś [✉]
Department of Migratory Fishes in Rutki
Inland Fisheries Institute in Olsztyn, 83-330 Żukowo, Poland
e-mail: rber@infish.com.pl

R. Wenne
Institute of Oceanology, Polish Academy of Sciences
Powstańców Warszawy 55, 81-712 Sopot, Poland

Introduction

In theory, stock enhancement with cultured fish has three primary goals: restoring species, increasing the numbers of natural populations, and sea ranching (Bell et al. 2008). Baltic countries enhance populations of migratory sea trout, *Salmo trutta* L., with the aim of compensating for the lack of sufficient spawning grounds that have been lost to anthropogenic pressure including infrastructure construction on rivers, stream flow regulation, overfishing, and water pollution. Cultured salmonids are routinely released into the natural environment where their gene pool mixes with that of the wild fish with which they interact. The genetic impact of cultured fish on wild populations is well documented and results in, *inter alia*, reduced genetic polymorphism and disrupted population genetic structure or selection

(e.g., Hindar et al. 1991, Waples 1991, Ryman et al. 1995, Naish et al. 2007). In general, cultured fish are characterized by lower condition index values than wild fish, and their reproduction is usually less successful (Seamons et al. 2012, Christie et al. 2014). Since stock enhancement programs can help to protect endangered species while simultaneously causing a variety of ecological and evolutionary threats (e.g., Fraser 2008, Neff et al. 2011), it is paramount to consider very carefully the aim of stock enhancement (and to determine the advantages and risks) and the methods used to implement it (Naish et al. 2007).

In Poland, supplementing natural sea trout stocks dates to the nineteenth century (Kołder 1958). Regular stock enhancement in the Vistula basin and Pomeranian rivers has been conducted since the 1960s (HELCOM 2011). The quantities of smolts released in the Baltic region has been fairly stable since the end of the 1980s at an average of 2.97 million individuals in the 1988-2015 period (ICES 2016). Poland, Finland, and Sweden have operated the largest stock enhancement programs, and the Polish contribution in the 2000-2015 period is estimated to be from 0.8 to 1.5 million smolts annually (ICES 2016). In most Baltic countries stock enhancement is based on hatchery production using material obtained from wild spawners caught in native rivers during spawning migrations. In Poland, this is only possible with the populations in Pomeranian rivers. Enhancement programs in Vistula basin rivers has been conducted, especially in recent years, mainly with material from cultured broodstocks, because of difficulties in obtaining sufficient quantities of eggs from sea trout returning to the Vistula. The required quantities of eggs are thus supplemented with those obtained from cultured broodstocks. The source of material produced in hatcheries is a significant issue, and the current situation is certainly not advantageous (Wąs and Bernaś 2016), especially since more than half of Polish sea trout enhancement is done in the Vistula basin (ICES 2016). In theory, cultured sea trout broodstocks (Aquamar, Dąbie and Rutki) are supposed to be a genetic reservoir that is refreshed with newly introduced genotypes from fish caught during spawning migrations, and production in hatcheries is based mainly on sea trout returning to rivers naturally, as is the case in other Baltic countries.

The share of cultured sea trout compared to that from natural spawning in Polish rivers is unknown. Partial information is available regarding the sea trout in Pomeranian rivers, which was obtained based on adipose fin clippings, and it reveals that the quantity of fish originating from smolt stocking as compared to the rest of the spawning stock ranges from about 15 to over 70% (Bartel et al. 2009, Bernaś 2014). This data, however, does not take into consideration the share of individuals originating from alevins and fry stocking, but only those from smolt stocking. In the case of the Vistula, the quantities of wild and cultured sea trout is not known. Given the availability of spawning grounds and the level of stock enhancement, one can assume that the share of cultured fish is high. It is assumed that a substantial portion of sea trout in Polish rivers originate from artificial reproduction; however, because of difficulties associated with methods of identifying cultured fish, assessing the effectiveness of stock enhancement programs is currently very difficult. In the second half of the twentieth century, the most common marking method for Baltic salmonids was either the external Carlin tag or the Floy Tag. This type of marking is still used, but on a smaller scale, because of successively diminishing returns, which currently do not permit assessing effectiveness. Additionally, this method is limited to larger juveniles, for example, smolts (Nielsen 1992). Marking methods for larvae or fry include immersion in fluorescent dyes (Secor et al. 1991, Jones et al. 2005), using transgenerational enriched stable isotopes that appear in bone tissue (Munro et al. 2009), or chemicals such as oxytetracycline (Krumme and Bingel 2016). Besides the obvious advantages of these methods, they do not deliver individual information and often require sacrificing the individual fish caught, which is also why they are not used to assess stock enhancement effectiveness.

A new marking system was introduced in the early twenty-first century based on clipping smolt adipose fins. This method is used widely with cultured

salmonids in the USA, Canada, and in Europe. In the Baltic basin, this method is obligatory in for all cultured salmon, *Salmo salar* L. and sea trout smolts in Sweden and Estonia, while in Finland, Denmark, and Russia it is required for a portion of the cultured population. In Poland since 2006, all sea trout smolts released into Pomeranian rivers have clipped adipose fins; however, to date it has not been possible to implement this marking method with all of the sea trout introduced into the Vistula. Despite a range of advantages, clipping the adipose fin also has drawbacks. The first, and most significant, is that it provides no information on the effectiveness of stocking with alevins and fry, which, in some areas, comprises most of the material released. Additionally, this method is labor intensive, increases fish mortality stemming from the wounds and abrasions the fish suffer during the procedure, and poses certain ethical concerns.

These disadvantages are lacking in molecular methods that have been developed recently that use highly polymorphic loci, for example microsatellite DNA. This method is based on identifying genotypes that occur naturally and analyzing their variability (Estoup et al. 1998). This method reduces the mortality of smolts used for stock enhancement to the minimum, because the material for genetic analysis is collected *in vivo* only from mature fish used in artificial spawning. Genetic testing is advantageous for the fish because of its non-invasive nature, but there is also a range of logistical benefits. The number of fish required for the analysis of stock enhancement effectiveness is reduced. Only the spawners used in artificial reproduction and a small number of fish returning to rivers after several years are subjected to genetic marker analysis. Using a set of about a dozen genetic markers it is possible to identify individuals and to conduct parentage analysis that either confirms or excludes kinship between a given parental pair and alleged offspring. Genetic methods have yet another important advantage, namely increased assessment precision. This method permits determining whether an individual originates from artificial or natural spawning regardless of the type of material (alevins, fry, or smolt) the specimen came from. There is also greater precision with this method since it can determine the effectiveness of the material from a given facility or farm producer. Finally, this is the only method that can monitor whether stock enhancement is preserving genetic biodiversity and maintaining the population being enhanced at an appropriate level of genetic diversity, which is currently something to which great importance is attached (Wąs and Wenne 2002, 2003, Bernaś et al. 2014, Wąs and Bernaś 2016, Wenne et al. 2016). The usefulness of genetic methods in analyzing parentage has been confirmed in many species including Atlantic salmon, rainbow trout *Oncorhynchus mykiss* (Wal.), Atlantic cod *Gadus morhua* L., and carp *Cyprinus carp* L. (Norris and Cunningham 2004, Fishback et al. 2002, Vandeputte et al. 2004, Herlin et al. 2007). Simultaneously, the performance of genetic methods has improved considerably recently with the development of statistical methods (Jones et al. 2010).

The aim of this paper is to propose a strategy for assessing the effectiveness of stock enhancement in the Vistula with genetic methods. A set of genetic markers is presented that is useful in identifying migratory sea trout from artificial and natural reproduction, and the parameters are designated that should be used in analyses for determining kinship with the algorithms in the Family Assignment Program (FAP) and SOLOMON.

Material and methods

To test the usefulness of microsatellite loci for identifying Vistula sea trout individuals, small samples (4 mm^2 each) were collected from the tail fins of 144 Vistula sea trout spawners (group T2001) that were used in artificial reproduction in 2001 in Świbno (122 females and 22 males) and from 172 individuals caught in 2003 in the Vistula mouth in Świbno (group T2003). The fish examined were mature specimens and most had sea age 1+ and 2+. The trout caught in 2003 could not be the offspring of the sampled spawners used in 2001 and were used in the analyses

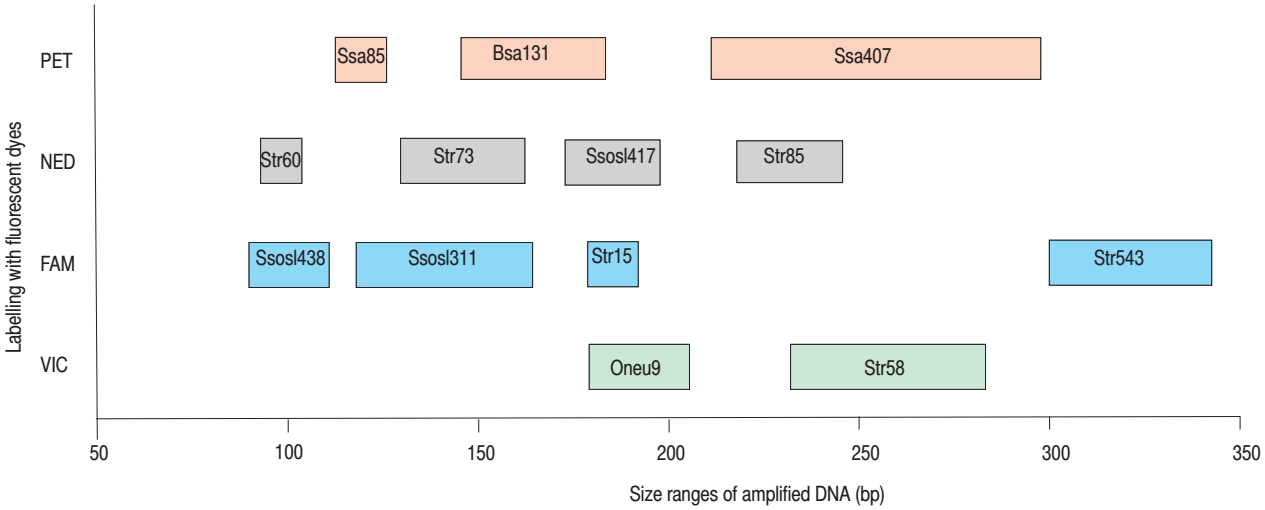

Figure 1. A set of 13 fluorescent-labeled microsatellite markers in relation with length range of the amplified DNA fragments designed for the analysis of multiplex-PCR. The colors red, grey, blue, and green correspond to fluorescent labeling PET, NED, FAM and VIC, respectively.

as negative controls. DNA was isolated with the Genomic Mini kit according to the manufacturer's protocol (A&A Biotechnology, catalog no. 116-250). A set of 13 microsatellite loci were used to identify the individuals as follows: *Ssosl438*, *Ssosl311*, *Str15INRA*, *Str543INRA*, *OneU9*, *Strutta58*, *Str60INRA*, *Str73INRA*, *Ssosl417*, *Str85INRA*, *Ssa85*, *Bsa131*, *Ssa407* (Cairney et al. 2000, Estoup et al. 1993, O'Reilly et al. 1996, Poteaux et al. 1999, Presa and Guyomard 1996, Scribner et al. 1996, Slettan et al. 1995, Slettan et al. 1996). The DNA of the loci was amplified during the polymerase chain reaction (multiplex-PCR) with a QIAGEN Multiplex PCR kit (QIAGEN, catalog no. 206145). The reaction mixture of a volume of 9 μl was prepared according to the standard Multiplex-PCR protocol (final concentration : 1 x Master Mix, from 0.1 to 0.2 uM of each PCR reaction primer) and supplemented with 1 μl of DNA solution (30-100 ug ul^{-1}). The reaction was conducted in a TProfessional Basic Gradient thermocycler (Biometra) at the following temperature profiles: 95°C 15 min/denaturation, 38 cycles for (94°C – 30 sec/denaturation, 55°C – 1 min/annealing, 72°C – 1 min/chain extension), and 60°C – 30 min/final chain extension. Thanks to the fluorescent labeling (VIC, FAM, NED, PET) of PCR primers, genotyping the amplified markers was also performed simultaneously using an ABI3730 automated sequencer. The length range of the DNA fragments of the different loci was considered when choosing the fluorescent labels so that they did not overlap (Fig. 1). Assessing genetic polymorphism and kinship in the parent/offspring lines among the sea trout groups analyzed was performed using GenAlEx 6.5 (Peakall and Smouse 2012), FAP (Family Assignment Program) (Taggart 2007) and SOLOMON (Christie et al. 2013). FAP is an exclusion-based method program that generates three types of information: (1) the prediction power of a given panel of loci for assigning offspring to parental pairs; (2) the actual assignment of offspring to parental pairs; and (3) the identification of potentially problematic loci. This algorithm is best suited for cases when all of the parents of the offspring in the parental candidate pool are known and there are no null alleles (Taggart 2007). SOLOMON uses Bayesian method to analyze kinship. To identify true parent-offspring pairs, it determines the posterior probability that the assumed parental pair is false based on the frequency of common alleles. Calculations were performed according to recommendations for microsatellite loci: 1,000 simulated data sets for 50,000,000 simulated genotypes (Christie et al. 2013).

Table 1
Fluorescent labeling and characteristics of variation of the analyzed markers, size range of the amplified DNA fragments in base pairs (bp), number of observed alleles, Ho – observed heterozygosity, PIC – Polymorphism Information Content

Locus	Size range (bp)	Fluorescent labeling	Number of alleles	Ho	PIC
Oneu9	187 - 205	VIC	8	0.24	0.22
Str58	232 - 281	VIC	20	0.89	0.88
Ssosl438	92 - 106	FAM	9	0.44	0.4
Ssosl311	124 - 158	FAM	18	0.89	0.85
Str15	177 - 187	FAM	6	0.73	0.68
Str543	296 - 346	FAM	14	0.69	0.65
Str60	89 - 103	NED	4	0.48	0.36
Str73	138 - 144	NED	7	0.59	0.53
Ssos417	173 - 193	NED	11	0.82	0.78
Str85	218 - 244	NED	8	0.62	0.51
Ssa85	112 - 128	PET	8	0.72	0.67
Bsa131	147 - 183	PET	9	0.76	0.74
Ssa407	209 - 291	PET	35	0.94	0.93

Results

The designated set of 13 microsatellite markers was highly selective. All of the individuals tested had individual genotypes that were clearly distinguishable. The values of the polymorphic coefficient (PIC) for individual loci ranged from 0.22 to 0.93 (Table 1). The length of the analyzed microsatellite fragments of DNA for the 13 loci was characterized by a large span of 90 to 342 base pairs (Table 1). There were from 4 to 35 alleles throughout the pool of sea trout examined (a total of 157 alleles) for each loci. The heterozygosity level ranged from 95% to 26% for loci *Ssa407* and *Oneu9*, respectively (Table 1). Importantly, the null allele phenomenon, which is common with microsatellite markers, was not observed among the loci analyzed. This confirmed the usefulness of the designated panel of 13 loci for the proposed kinship assignment. The effect of null alleles is appeared when one of the alleles is not read in the diploid genotype because of reduced efficiency in the amplification process. In this case, genotypes could have been incorrectly identified, which would resulted in incorrect kinship assignment. Negative control was used to confirm that the set of markers used in the parentage analysis was sufficiently selective. The group of Vistula sea trout spawners from 2001 (T2001 – simulated parental individuals) and mature individuals returning to the Vistula to spawn in 2003 (T2003 – simulated offspring group) should presumably show a total lack of direct parent/offspring kinship. In a comparison of the parental genotype (T2001) with that of the assumed offspring (T2003) using the panel of 13 markers without considering the genotyping error threshold, FAP indicated a 100% lack of kinship between the two groups. The same result was obtained when a single mismatched allele in genotype (parameters recommended by the author of Taggart 2007) was included in the analysis.

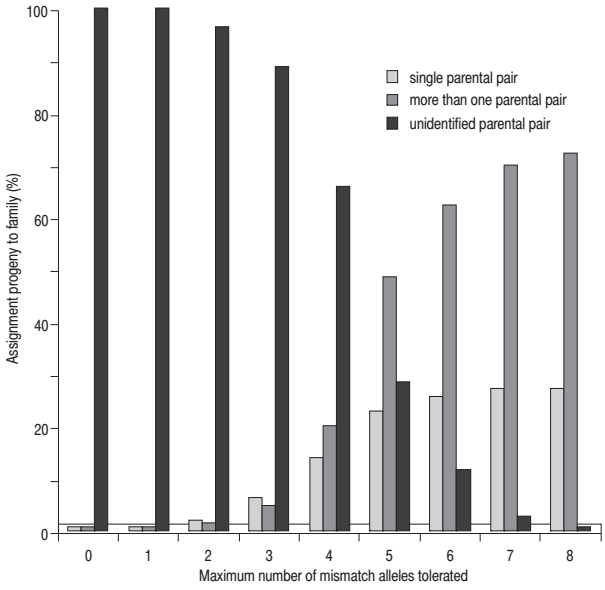

Figure 2. Parentage assignment of presumed offspring to parent group computed in FAP for the panel of 13 loci, depending on the number of mismatched alleles tolerated.

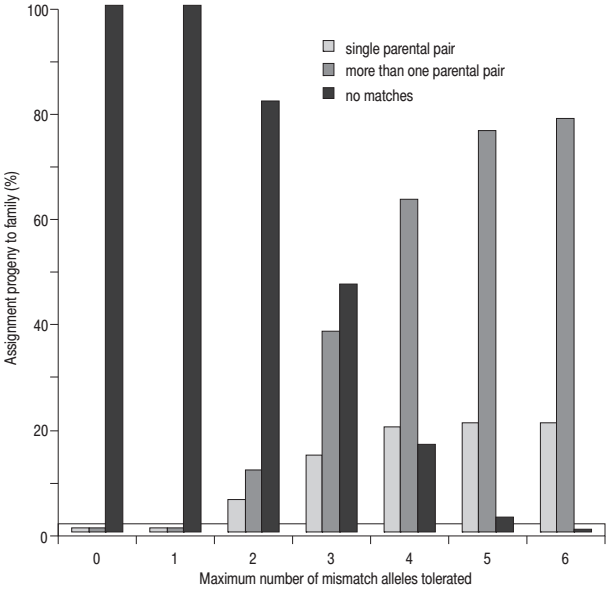

Figure 4. Parental assignment of presumed offspring to parental group for the eight most polymorphic loci, depending on the number of mismatched alleles tolerated.

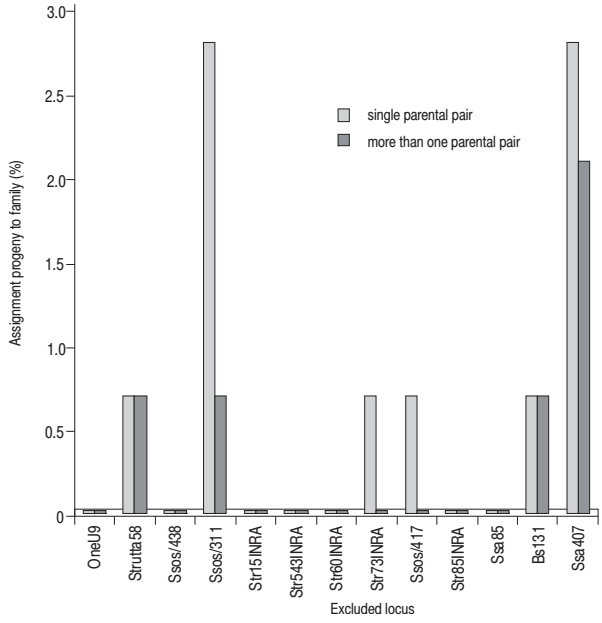

Figure 3. Parental assignment of presumed offspring to parent group after eliminating one locus from the panel of 13 markers.

Assuming two erroneous alleles, at least one parent pair was assigned to 3.5% of the 2003 spawners (Fig. 2). When three to six erroneous alleles were assumed, increasingly higher parent/offspring kinship values were obtained (11.1, 33.4, 69.4, and as high as 88.2%, respectively). However, the kinship assigned was only the result of simulation, because the sea trout returning to the Vistula in 2003 could not have been the offspring of the sea trout spawners used in artificial reproduction in 2001. Allocation analysis (parental pair assignment) was also conducted with a reduced number of markers. During these tests, a single allele mismatch tolerance was considered. Eliminating just a single locus, depending on its polymorphism, resulted in the erroneous assignment of from 0 to 4.9% of unrelated individuals to the parental group (Fig. 3). By excluding the subsequent 2, 3, 4, and 5 loci being sequenced and testing all possible combinations of them, a set of the eight most polymorphic loci was identified that are essential for obtaining 100% non-kinship between the groups analyzed. These were loci *Strutta58, Ssosl311, Str15INRA, Str543 INRA, Str73INRA, Ssosl417, Bs131*, and *Ssa407*. Of course, with this set of markers the kinship assignment of parental pairs was > 0 at a genotyping error threshold > 1 (18.1, 52.8, 83.3, and 99.3% with 2, 3, 4, 5, and 6 mismatches in genotyping (Fig. 4)). However, if the analysis was performed assuming the same error threshold for

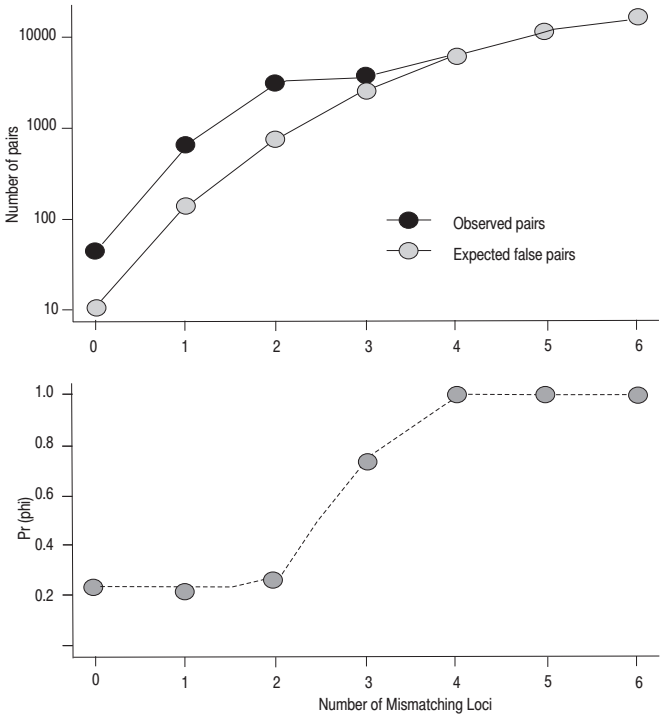

Figure 5. Parentage assignment computed in SOLOMON for 13 loci depending on the number of mismatched alleles tolerated. Upper graph illustrates the number of pairs observed and the expected number of false pairs, or the number of pairs that should occur by chance. The upper surface is on a scale of $\log^{10}$. Pr (phi) equals the number of expected false pairs divided by the total number of pairs observed (incorrect for a given number of loci) and is displayed in the lower graph.

13 and eight loci, the level of erroneous kinship assignment of parental pairs was higher with eight loci. These results demonstrated conclusively that increasing genotype error above 1 reduced analysis selectivity and reliability, and it should not be done. It was possible, though, to limit the number of analyzed microsatellite loci required for parentage analysis to the essential eight most polymorphic. However, this is invalid if all 13 markers can be analyzed simultaneously. When using all the loci, Bayesian calculations also did not indicate any parental pairs for either 1 or 2 unmatched loci (probability of parental assignment < 0.05). Significantly, the number of expected and observed false positive pairs was comparable, and the results of kinship assignment were identical with the analysis based on Mendelian distribution in FAP (Fig. 5).

Discussion

In the Baltic basin marking sea trout and salmon is mainly done with conventional tags (Carlin tags and FloyTag) and by clipping the adipose fin. Unfortunately, the number of returns from external marking is decreasing consistently, and changing the marking system is currently under discussion (ICES 2016). Simultaneously, the number of salmon and sea trout smolts with clipped adipose fins observed is on the rise. In 2015, approximately 2.5 million salmon smolts and 800,000 sea trout smolts were marked with this method in the Baltic basin. In Poland, this marking method has been used in Pomeranian rivers since 2006; however, in response to warnings from veterinarians, this type of marking was halted in 2013 and 2014 (ICES 2016). Currently, the question has been resolved at least in the Vistula catchment, where this method is not used. In the Baltic basin, genetic methods are used primarily to identify origin, which means determining to which stock or population an individual belongs (mixed stock identification) (e.g., Koljonen 2006). Studies focused on identifying individuals from either artificial or natural spawning are performed much less frequently. This type of research has, for example, been done in Ireland using historical salmon data (Aykanat et al. 2014) or on Pacific salmon (e.g., Ford and Williamson 2010, Hess et al. 2012). The possibility of assessing stock enhancement effectiveness precisely is significant to sea trout resource management. The genetic method discussed in this paper appears to be the most advantageous of those used to date for identifying fish from artificial spawning and for assessing stock enhancement effectiveness in the Vistula. The results indicate that the designated microsatellite panel is characterized by high selectivity. All of the specimens examined had individual genotypes, which permitted assigning them precisely. The number of alleles for the 13 loci presented in the pool of individuals tested was 157, which, theoretically, meant that 13 billion complex genotype combinations were possible. The possibility of identifying individuals so precisely with

Table 2
Example of the sampling and analysis in the context of genetic methods for assessing sea trout stocking efficiency in the Vistula River

	Years							
	2017	2018	2019	2020	2021	2022	2023	2024
Source	No. of analyzed individuals							
Miastko	1200	180	180	180	180	180	180	180
Dąbie	400	60	60	60	60	60	60	60
Rutki	200	30	30	30	30	30	30	30
Lubicz	350	350	350	350	350	350	350	350
Świbno	250	250	250	250	250	250	250	250
Σ	2400	870	870	870	870	870	870	870

the simultaneous lack of the null allele phenomenon among the analyzed loci confirmed the utility of the designated panel of markers for identifying specimens and determining parent/offspring kinship, and, thus, this is linked with assessing stock enhancement effectiveness. The parental pair assignment simulations conducted with negative controls (the offspring group did not originate from the parental group) indicated unambiguously that decreasing the number of loci analyzed to less than 13 or increasing the mismatched alleles tolerance above a value of 1 decreased the selectivity of the designated panel and could generate false, inflated kinship assignment. Therefore, using 13 markers and setting the error threshold at 1 in FAP was the optimum solution.

There are a variety of parentage analysis methods based on genetic polymorphism. Generally, exclusion-based and likelihood-based methods, with a few modifications, are used in parentage analysis (Jones et al. 2010). Exclusion-based methods are very simple and do not usually introduce hypotheses other than Mendelian inheritance, but they are very sensitive to genotyping errors. When errors are moderate and the theoretical assignment power is high, the problem of genotyping errors can be solved by permitting a small number of mismatched alleles between offspring and parents (Vandeputte et al. 2006). The most frequently used exclusion method programs in aquaculture are VITASSIGN (Vandeputte et al. 2006) and FAP (Taggart 2007). Methods based on likelihood use quantitative Mendelian inheritance to calculate probability in various candidate relationships within a set, and then the relationships with the greatest inferred probability are selected. The most popular programs with similar algorithms are Cervus (Marshall et al. 1998), Colony (Wang 2004), PAPA (Duchesne et al. 2002), and SOLOMON (Christie et al. 2013). When choosing an appropriate model for parentage analysis, several questions must be answered, most importantly what is the aim of the analysis and what kind of data sets are available. In the case of the present research, the data included all the genotypes of all the parents used in artificial spawning, and the number of offspring was large (>10), it seemed that algorithms based on exclusion (e.g., FAP), likelihood (e.g., CERVUS), or Bayesian methods (SOLOMON) would be sufficient, particularly when used together (Jones et al. 2010). As the results of the comparative simulation indicated, the likelihood method complements the exclusion method well and its effectiveness is at least as high.

As practice indicates, it was not possible to fully implement the adipose clipping system with Vistula sea trout, while this method was put on hold for a period of time with Pomeranian sea trout. Thus, we

recommend using methods that employ genetic markers and propose a plan for cyclical assessments of stock enhancement effectiveness in the Vistula. This would be based on annual genetic analyses of several hundred parent pairs used to produce material (Table 2). The tests would be conducted, most importantly, on spawners caught during the fall in the Vistula and its tributaries that are used to produce material in hatcheries. Over the past several years, from 300 to 600 fish have been caught annually for this purpose. Simultaneously, some of the cultured sea trout used for artificial reproduction in a given year would be subjected to genetic analysis, which would, however, be organized slightly differently so as to reduce costs as much as possible and to limit interfering with the well-being of the stock. Cultured sea trout used in the production of material in hatcheries would be marked with, for example, passive integrated transponders (PIT tags) in addition to the genetic analysis performed to determine the genotype of specific individuals (a genetically defined parental base). In subsequent years, the only fish that had spawned in a given year would be reported. This way, only in the first year of implementing the research/project would genetic analysis be conducted on a larger group of fish that would include all cultured specimens to be used in hatcheries for the production of material in subsequent years. Three hatcheries (in Miastko, Dąbie, and Rutki) produce stocking material for enhancement programs conducted on the Vistula basin. Thus, approximately 2,000 fish would have to be marked and subjected to genetic analysis to create the genotype base. Assuming that, annually, only a small percentage of the broodstock is used for the first time for artificial reproduction (first-time spawners), it would also be necessary to successively mark and genotype these fish. Assigning kinship between fish returning to rivers (presumed offspring) and parent specimens used in artificial reproduction would require analyzing approximately 300 of the fish returning to rivers annually, and this group would be the spawners caught annually in rivers and used in the production of hatchery material. This would minimize the number of specimens that would have to be subjected to genetic analysis. Assessing enhancement effectiveness would be delayed by three to four years, as was with other assessment methods because of the lag time required to obtain biological material from returning fish.

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