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Title: COMPAS-PCR method and methods for detecting, identifying or monitoring salmonid species and/or gender

FIELD OF THE INVENTION

The present invention provides an asymmetric PCR method, the COMplementary-Primer-Asymmetric (COMPAS)-PCR, and specific methods for detecting, identifying or monitoring the species and/or gender of salmonids. The present invention also encompasses oligonucleotide primers corresponding to species specific sequences. The use of the methods and primers are also an aspect of the present invention together with kits comprising said primers.

BACKGROUND OF THE INVENTION

Since the Polymerase Chain Reaction (PCR) was invented by Kary Mullis in the mid-80s [1], Nucleic Acid (NA) amplification techniques have had an unprecedented development for molecular biology applications. A contributing factor to this success is its flexibility with the development of several modifications which expands the technical capabilities of PCR. In particular several methods have been developed for the detection of point mutations such as the Amplification Refractory Mutation System (ARMS) [2] and variants such as the PCR Amplification of Specific Allels (PASA) [3, 4], bidirectional-PASA [5] or Mistmatch Amplification Mutation Assay (MAMA) [6], Taq-MAMA [7] and Melt-MAMA [8]. Point mutations, also called Single Nucleotide Polymorphism (SNP), constitute differentiating genetic information which may be relevant in various contexts spanning from medical applications for disease diagnostics to population genetics and species identification. As the amount of PCR application increases, limitations inherent to DNA chemistry may become more challenging. For instance primer complementarity leading to primer dimer formation, is a limiting factor for the design of generic PCR.

It is believed that the asymmetric PCR method according to the present invention will alleviate PCR limitations due to primer complementarity incompatibility. Said method was further applied for the development of a PCR assay for direct repeat sequences.

NA technology can be characterized as noninvasive since very little subject sample is required for obtaining sufficient NA for analysis. The fish farming industry is in need for such noninvasive methods for the identification of species and gender of salmonids and in particular *Salmo salar* (Atlantic salmon) and *Salmo trutta* (brown trout) and their hybrids.

Methods for species determination and inter-species hybridization between different salmonid fish species is an important tool in ecological studies, and when assessing the impact of aquaculture escapees on indigenous populations. Ecological studies have shown that inter species hybridization can severely impact population size and viability estimates. Morphological discrimination between hybrids and parental species is in many cases difficult

(e.g. *S. salar* X *S. trutta*), and the behavior of the hybrids may be different from that of the parental species [9].

Genetic gender determination without terminal sampling (i.e. killing the fish) is a valuable tool in ecological studies using telemetry. Fish gender behavioral differences are an often overlooked parameter in assessing the population vulnerability towards environmental perturbations, i.e. in anadromous brown trout (*salmo trutta*), there is a marked gender-bias in anadromity, which render the females (i.e. the ecologically relevant reproductive part of the population) more susceptible to marine environmental factors than males.

There is also a large commercial potential for genetic gender determination in the aquaculture industry. The breeding companies that deliver fish eggs from selected stocks currently rely on ultrasound- sex determination when fish are 1-2 kg. This method is laborious and costly. Genetic gender determination at an early stage will enable the companies to reduce female production cost by early elimination of males, hence eliminating the associated aquaculture cost.

Identification of fish species by PCR has previously been addressed and solutions have been suggested. However, when selecting available methods for identification of salmonid species as e.g. in References [10-14] , the method published by A. M. Pendas et al., (Chromosomal mapping and nucleotide sequence of two tandem repeats of Atlantic salmon 5 S rDNA Cytogenet Cell genet 67:31-36 (1994)) [14] seemed to give the best result among the methods tested. The inventor surprisingly discovered that said method was not reliable for the above mentioned purpose as the identification of the species *Salmo salar* and *Salmo trutta* were not distinguished satisfactorily using the PCR method and primers described in[14]. It is believed that the present invention provides a robust simple PCR method capable of specifically identify *Salmo salar* and preferably differentiate between *Salmo Salar, Salmo trutta* and their hybrids. The inventor tested the Salmo-A & B PCR method by A.M. Pendas et al.; the primers were originally designed based on the 5S rDNA sequence of the rainbow trout (*Salmo gairdnerii*, renamed *Oncorhynchus mykiss*) [15] and used on *Salmo trutta* and *Salmo salar* among other fish species [16]. These primers amplify 118bp of the 120bp coding sequence of the 5S rDNA together with the associated variable non coding sequence. The amplification product(s) varies in length and were used to differentiate close species. Two loci were originally found to be amplified for *Salmo salar*, one major product about 255bp and a minor product around 525bp. These results are corroborated by the 2 published sequences for *Salmo salar* gb S73107.1 & gb S73106.1. Similarly, *Salmo trutta* also showed a double band pattern but with longer products both for the major and minor loci [14]. Hybrids between *Salmo salar* and *Salmo trutta* were found to produce both product types for the major loci around 255bp and longer respectively [16]. No product(s) difference between *Salmo salar*

and for *Salmo trutta* was detected by the present inventor using the method of A.M. Pendas et al., to differentiate *Salmo salar* from *Salmo trutta* and their hybrids. However, the genetic target of said PCR method, the 5S-rDNA, was used to develop the novel and inventive method together with specific oligonucleotide primers of the present invention.

Most of the work for identifying gender associated genetic markers among salmonids have used the rainbow trout or the Chinook salmon as model organisms [17-25]. Several of these targets were unsuccessfully tested by the inventor on *Salmo salar* in an effort to differentiate females from males by PCR.

In August 2012, Yano et al., published the Master Sex-Determining Gene ”sdY” in the rainbow trout [26]. The primers described by Yano et al., where then applied by the inventor to further develop a specific gender PCR method able to identify the gender in *Salmo salar* and *Salmo trutta*. It is believed that the method according to the present invention will be a more reliable test for identification of gender in salmonids, preferably in *Salmo salar* and *Salmo trutta.*
It is further believed that both identification of gender and identification of species as described above are possible to achieve in one single test.

SUMMARY OF THE INVENTION

The present invention encompasses in a first aspect a method of asymmetric PCR comprising:
- providing a nucleic acid sample to be used as a target template;
- identifying target template(s)
- performing a polymerase chain reaction, utilizing highly complementary primers wherein either the forward or the reverse primer concentration is decreased to unblock the PCR reaction by initially promoting linear amplification which will progressively shift towards exponential amplification by the COMplementary-Primer-ASymmetric (COMPAS)-PCR;
- - identifying the amplified nucleotide target sequence(s);

A second aspect of the present invention comprises a method for detecting, identification or monitoring salmonid species comprising:
- providing a nucleic acid sample from salmonid to be used as (a) target template(s);
- performing a polymerase chain reaction (PCR) applying COMplementary-Primer-ASymetric (COMPAS)-PCR of the present invention, to amplify a nucleic acid target sequence of the template (s), utilizing a set or several sets of highly complementary primer pair(s) capable of priming said target(s);
- identifying the amplified nucleotide target sequence(s);

- determining the species.

In a third aspect the present invention provides oligonucleotide primers, selected from the oligonucleotides of Tables 1 or 2 or any combinations thereof, or oligonucleotides with complementary sequences or functional equivalent sequences.

In a fourth aspect the present invention comprises a method for the determination of salmonid gender comprising:

- providing a nucleic acid sample from salmonid to be used as (a) target template(s);
- performing a 2-step duplex real time polymerase chain reaction (qPCR), to amplify a nucleic acid target sequence of the template (s), utilizing a set or several sets of primer pair(s) selected from Table 3,
- identifying the amplified nucleotide target sequence(s);
- determining the gender by a melting curve analysis of the PCR product.

In a fifth aspect the present invention comprises a method for detecting and identification of salmonid species and/or gender, comprising the method of the present invention and High Resolution Melt analysis, wherein the primer sets are selected from Tables 1, 2 and/or 3 or complementary sequences thereof, in any combinations.

Further it is provided in a sixth aspect a kit for detecting and identification of salmonid species, comprising a collection of oligonucleotide primers selected from Tables 1 and 2 in any combinations or complementary sequences thereof, capable of detecting salmonid species by the method of the present invention.

A seventh aspect of the present invention encompasses a kit for detecting and identification of salmonid species and/or gender comprising a collection of primers selected from Tables 1, 2 and/or 3, in any combinations, or complementary sequences thereof capable of detecting salmonid species and/or gender by the method of the present invention.

Finally in further aspects the present invention comprises use of the methods of the present invention, or the oligonucleotide primers of the present invention or use of the kits provided from the present invention.

Preferred embodiments are set forth in the dependent claims and in the detailed description of the invention.

DESCRIPTION OF THE FIGURES

Figure 1. Illustrates COMPAS-PCR optimalisation example for *Salmo salar* & *Salmo trutta,* varying the concentration of the forward primer 5SNTS-23F from 0.6 to 0.05µM while using a constant concentration of 0.6µM for the reverse primer 5SNTS-22R+2.

Figure 2. Illustrates a simplified diagram of the direct repeat-sequence COMPAS-PCR and in particular applied to the identification of species method.

Figure 3. Illustrates a detailed diagram of the identification of species method. All tested primers are shown by the arrows. "Salmo salar specific" shows the specific reverse primers with 3 nucleotide "overhang" in the NTS section.

Figure 4. Illustrates specific amplification differentiation between *Salmo salar* & *Salmo trutta,* using a reverse primer with 1 additional nucleotide in 3' compared with Fig 3: 5SNTS-23R+3. Forward and reverse primer concentrations are as described in Fig 3.

Figure 5. Illustrates amplification curves for the duplex qPCR performed on *S. salar*, *S. trutta* and hybrids for differentiating males from females based on sdY detection. 18S is used as a positive control.

Figure 6. Illustrates melt curve analysis for the duplex qPCR performed on *S. salar*, *S. trutta* and hybrids for differentiating males from females based on sdY detection. 18S is used as a positive control

Figure 7. Illustrates the identification of species method primers. The primer pair 5SNTS23F + 5SNTS23R+3 is indicated by the arrows. Note: All reverse primers are shown as their reverse complement.

Figure 8. Illustrates results for the identification of species method as described in Example 2.

DETAILED DESCRIPTION OF THE INVENTION

It is an object for the present invention to provide an asymmetric PCR method and non-invasive methods for identification of species and/or gender among salmonids, preferably *Salmo salar* and *Salmo trutta* and their hybrids, using almost complementary primers targeting direct tandem repeats. The present invention also encompasses oligonucleotide primers corresponding to

species specific sequences or single nucleotide polymorphism (SNP). The use of the method and primers are also an aspect of the present invention together with kits comprising said primers. In order to unlock complementary primers for target product amplification, an asymmetric PCR method was developed by decreasing either the forward or the reverse primer concentration until optimal PCR amplification is reached. As shown in Figure 1, this has progressively generated strong and equal amplification (unspecific) for both *S. salar* and *S. trutta* as the forward primer concentration was decreased. Asymmetric PCR has been previously described for enhancing probe based detection during which the PCR will shift from exponential to linear amplification to favor probe hybridization to its target single stranded sequence, a method called Linear-After-The-Exponential (LATE)-PCR [27, 28]. In the present invention, where the primers used are highly complementary, the asymmetric PCR has an opposite pattern shifting from linear to exponential amplification, effectively alleviating the target amplification inhibition otherwise observed with complementary primers (see Fig 1). During the first amplification cycles the concentration-limited primer will be mainly sequestered by the excess primer such that mostly excess primer linear amplification will take place. As linear product accumulates, target sequence concentration for the limiting primer increases, sequestering excess complementary primer concentration decreases therefore enhancing target priming and subsequent exponential amplification. We refer to said PCR method as COMplementary-Primer-ASymetric (COMPAS)-PCR. The given salmonid examples (Examples 2 and 4) use complementary primers which also have the same target sequence, but it is foreseen that the method may be applied to partly complementary primers having distinct target sequences.

In order to identify nucleotide sequences suitable as a target for discerning between the species, the inventor made a structural study of the 5S-rDNA tandem direct repeats and noticed that any single section of the tandem direct repeat, covering a length typical for primers (i.e. 20bp), would be appropriate to design complementary primers structurally covering 1 or more products depending on the tandem direct repeat number (see Fig 2). It is a common understanding in the art, that when designed on non-tandem direct repeats DNA, complementary primers would amplify in opposite direction, failing to define and amplify a product. In addition, complementary primer pair priming will occur and compete with primer target priming and hence strongly inhibit target amplification.

To favor priming to the target DNA contra priming to the reverse primer, the inventor increased the forward 3' primer end by 2 nucleotides which happened to be GG (strong priming). Similarly the 3' end of the reverse primer was extended, to favor its priming to the target contra priming to the reverse primer.

The presence of genetic variations and its adequate exploitation is the key for developing a specific test. As published sequences did not provide appropriate genetic variation information for *S. salar* and *S. trutta*, the inventor systematically tested out reverse primers, incrementing by 1 nucleotide at the 3' end. This explored one by one the possibility for SNP. As a basis for the testing, the reverse primer was chosen, as this primer extended in the non-transcribed sequence (NTS), which is more prone to variations than the coding sequence in which the forward sequence was extending (See Fig 2 and 3). Following this modus operandi, the inventor observed that by extending the reverse primer in 3' by 3 nucleotides into the NTS produced marked assay specificity with strong *S. salar* amplification and very weak *S. trutta* amplification (See Fig 4). This specificity was conserved when the 5' end was shortened or elongated keeping the specific 3' "anchor" unchanged (See Fig 3). The observed residual amplification for *S. trutta* was further eliminated by increasing PCR stringency (higher annealing temperature, shorter amplification time and 2 step instead of 3 step PCR).
By using this approach the inventor developed a robust method and solved the problem of discerning between the Salmonids preferably *Salmo salar* and *Salmo trutta* and their hybrids, using the asymmetric PCR method according to the present invention, and almost complementary primers targeting 5S rDNA tandem direct repeats. The inventor has by using complementary primers also defeated a technical prejudice in the art (Reference:[29, 30].), as it has been shown by the present invention that complementary primers may be used as a valuable tool when applying the COMPAS-PCR method according to the present invention.

The method is not restricted to identification of fish species. Using almost fully complementary primers targeting the same sequence may also be applicable to any organism with tandem direct repeats DNA motifs of interest as target sequences. For example the 5S r-DNA tandem direct repeats is in essence found in all eukaryotic cells [31, 32] and could therefore be further used to develop specific complementary-primer assays for other taxon and species than salmonids. Further, the method will be useful for developing assays using primers targeting distinct sequences but generating primer-dimers due to part complementarity.

A method for identification of gender in salmonids is provided by the present invention by applying a gender duplex qPCR on samples from salmonids preferably on *Salmo salar* and *Salmo trutta* and hybrids and primers disclosed in Yano, A. et al [26].

The method by Yano, A. et al. [26], applied a regular PCR method and displayed the result on a gel. Said method was developed and tested on the Rainbow Trout, *Oncorhynchus mykiss*. There is however, also a need for gender differentiation for other salmonids species such as for *S. salar* and *S. trutta* as well as for the development of a faster, easier and more reliable test.

The inventor successfully developed the method for genetic gender determination of *Salmo salar* and *Salmo trutta.*

Moreover, the inventor improved the method using a real time PCR with a melt curve analysis, using parameters more stringent than usually used (i.e. 0,2°C increments instead of 0,5°C for the melt curve). The method of the present invention made it possible to clearly show and differentiate the two tops (indicating 2 products, i.e. male and female profiles, see Fig 6). When using 0,5°C increments the 2 tops could not be seen, and the gender could not be identified directly by qPCR. The method by Yano, A. et al. [26], used an annealing temperature of 60°C, with a 3 step amplification protocol lasting 90s total (30 + 30 +30). The annealing temperature of the present invention may be in the range of 60 °C to 65°C, and may be 62°C, more preferably 63°C, most preferably 64°C and the amplification may be a 2 step amplification protocol lasting preferably in 20s total (5 + 15). Also time spans of 10, 15, 25 and 30 seconds may be employed. This enables the amplification and the melt curve to be run in just about 1h to provide results without requiring any additional work i.e. running a gel.

Accordingly a first aspect of the present invention relates to a method of asymmetric PCR comprising:

- providing a nucleic acid sample to be used as a target template;
- identifying target template(s)
- performing a polymerase chain reaction, utilizing highly complementary primers wherein either the forward or the reverse primer concentration is decreased to unblock the PCR reaction by initially promoting linear amplification which will progressively shift towards exponential amplification by the COMplementary-Primer-ASymmetric (COMPAS)-PCR;

identifying the amplified nucleotide target sequence(s);

In one embodiment of this aspect the nucleic acid target sequence may comprise direct tandem repeats. In further embodiments the highly complementary primers may have a common overlapping DNA target sequence or the direct tandem repeat target may be in the 5s-rDNA region. Other regions may however, also be an option.

As used herein “complementary primers” refers to primers that are complementary to each other and will under previously known conditions bind to each other to form primer-dimers.

A second aspect of the present invention relates to a method for detecting, identification or monitoring salmonid species comprising:

- providing a nucleic acid sample from salmonid to be used as (a) target template(s);

- performing a polymerase chain reaction (PCR) applying COMplementary-Primer-ASymetric (COMPAS)-PCR of the present invention , to amplify a nucleic acid target sequence of the template (s), utilizing a set or several sets of highly complementary primer pair(s) capable of priming said target(s);
- identifying the amplified nucleotide target sequence(s);
- determining the species.

As used herein “salmonid” refers to a family of ray-finned fish. It includes salmon, trout, chars, freshwater whitefishes and graylings. The Atlantic salmon and trout of genus *Salmo* give the family and order their names.

In a further embodiment the determination of the species may be performed by a melting curve analysis of the PCR product or by electrophoresis analysis. Also other methods for determination of the PCR product may be employed.

In one or more embodiments the forward primer may be extended in the 3´ end to favor priming to the target and not to the reverse primer, the reverse primer may be extended in the 3´ end to favor priming to the target and not to the forward primer. The reverse or the forward primer may have a SNP at its 3’ end. Said primer may also be slipping out to the “right” in the 5S-rDNA coding sequence out of the complementary area with the reverse primer. Further the reverse primer may be extended in the 5´ end. The 3´forward primer may be extended by 4, preferably by 3, more preferably by 2 nucleotides.

In a further embodiment primers in the primer pair (s) are oligonucleotides each having a length of about 12 to about 30, preferably about 20 bp.

In a further embodiment the complementary set of primers may be selected from a set of primer pair (s), wherein the forward primer may be selected from Table 1 or a complementary sequence thereof and the reverse primer may be selected from Table 2 or a complementary sequence thereof or any combinations thereof.

The reverse primer may be locked at its 3´end at “+3” in the non-coding sequence, out of the complementary forward primer area, as the specificity lies in precise positioning of this SNP. At the other end, in 5’, the reverse primer may be shorter or longer (depends on the forward primer).

In one embodiment the salmonid of the present method may be *Salmo trutta, Salmo salar* and hybrids thereof. The method may however, be applicable to other salmonid species, other fish species or in fact any organism with tandem repeats DNA motifs.

In a third aspect the present invention comprises oligonucleotide primers, wich may be selected from the oligonucleotides of Tables 1 or 2 or any combinations thereof, or oligonucleotides with complementary sequences or functional equivalent sequences. The use of the oligonucleotides in a product, e.g a kit form furthers aspects of the present invention.

In a fourth aspect the present invention provides a method for the determination of salmonid gender comprising:

- providing a nucleic acid sample from salmonid to be used as (a) target template(s);
- performing a 2-step duplex real time polymerase chain reaction (qPCR), to amplify a nucleic acid target sequence of the template (s), utilizing a set or several sets of primer pair(s) selected from Table 3,
- identifying the amplified nucleotide target sequence(s);
- determining the gender by a melting curve analysis of the PCR product.

In a further embodiment of this aspect the increments of the melt curve analysis may be in the range of 0,1°C to 0,6°C, and may be 0,4°C, preferably 0,3°C, more preferably 0,2°C.

In further embodiments the annealing temperature may be in the range of 60°C to 65°C, and may be 62°C, more preferably 63°C, most preferably 64°C. The two step amplification may last for 30 sec., preferably 25 sec., more preferably 20 sec.

In a fifth aspect the present invention comprises a method for detecting and identification of salmonid species and/or gender, comprising the method of the present invention and High Resolution Melt analysis, wherein the primer sets may be selected from Tables 1, 2 and/or 3 or complementary sequences thereof, in any combinations.

In a sixth aspect the present invention provides a kit for detecting and identification of salmonid species, comprising a collection of oligonucleotide primers selected from Tables 1 and 2, in any combinations, or complementary sequences thereof, capable of detecting salmonid species by any one of the methods of the present invention.

In a seventh aspect the present invention provides a kit for detecting and identification of salmonid species and/or gender comprising a collection of primers selected from Tables 1, 2

and/or 3, in any combination, or complementary sequences thereof, capable of detecting salmonid species and/or gender by any one of the methods of the present invention.

Finally in further aspects the present invention comprises use of the methods of the present invention, or the oligonucleotide primers of the present invention, or use of the kits provided from the present invention.

Having now fully described the present invention in some detail by way of illustration and example for purpose of clarity of understanding, it will be obvious to one of ordinary skill in the art that same can be performed by modifying or changing the invention by using a wide and equivalent range of conditions and other parameters thereof, and that such modifications or changes are intended to be encompassed within the scope of the appended claims.

EXAMPLES

Example 1
COMPAS-PCR

Finn clips were collected from 1 *S. trutta* and 1 *S. salar* fish individuals for analysis. The samples were conserved in 98% EtOH prior to DNA extraction that was performed using mechanical and chemical methods for releasing PCR-grade DNA. Subsequent DNA measurement was performed using a nanodrop instrument (Thermo Scientific) and all samples were diluted in water to achieve a final concentration of 4 ng/µl. Samples (2.5 µl) were amplified in 25 µl final reaction mixtures using an ABI 7500 qPCR machine (Life technologies, Applied Biosystems). The mixture contained 12.5 µl MESA Blue qPCR MasterMix (Eurogentec), 0.6 µM reverse primer 5SNTS-23R+3, the forward primer 5SNTS-23F concentration was successively tested using: 0.6, 0.4, 0.2, 0.1 and 0.05 µM, the reaction was completed with distilled water, final volume of 25 µl. The 3-step PCR conditions consisted of 5 min activation at 95°C followed by 30 cycles of 95°C for 20 s, 62°C for 30 s and 72°C for 60 s. Real time amplification curves are shown in Fig1.

Example 2
Identification of species

Finn clips were collected from 4 *S. trutta* and 4 *S. salar* fish individuals for analysis. The samples were conserved in 98% EtOH prior to DNA extraction that was performed

using mechanical and chemical methods for releasing PCR-grade DNA. Subsequent DNA measurement was performed using a nanodrop instrument (Thermo Scientific) and all samples were diluted in water to achieve a final concentration of 4 ng/µl. Samples (2.5 µl) were amplified in 25 µl final reaction mixtures using an ABI 7500 qPCR machine (Life technologies, Applied Biosystems). The mixture contained 12.5 µl MESA Blue qPCR MasterMix (Eurogentec), 0.01 µM forward primer 5SNTS-23F, 0.6 µM reverse primer 5SNTS-23R+3 completed with distilled water. The 2-step PCR conditions consisted of 5 min activation at 95°C followed by 30 cycles of 95°C for 20 s and 72°C for 30 s. Products were visualized on 1,2 % agarose gels (Fermentas) stained with SybrGreen (See Fig 8).

Example 3

Identification of gender

S. salar fish samples were collected and processed for DNA extraction and measurement as described in Example 1. DNA Samples (2.5 µl) were amplified in 12.5 µl final reaction mixtures using a CFX-96 qPCR machine (Bio-Rad, Hercules, CA, USA). The mixture contained 7.5 µl SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA, USA), 0.2 µM forward primer sdYF, 0.2 µM reverse primer sdYR2, 0.05 µM forward primer 18SF and 0.05 µM reverse primer 18SR completed with distilled water. The 2-step duplex PCR conditions consisted of 2 min activation at 98°C followed by 40 cycles of 98°C for 5 s and 65°C for 15 s. Melt curve analysis was performed after amplification was completed by incrementing temperature stepwise by 0.2°C from 65°C to 95°C. Amplification curves and melt peak results are shown in Figure 5 and 6.

Example 4

Identification of species and gender

S. salar fish samples were collected and processed for DNA extraction and measurement as described in Example 1. DNA Samples (2.5 µl) were amplified as described in example 2, utilizing primers described in example 1 and 2, total 3 primer pairs. The triplex PCR amplification products were further analyzed by melt analysis as described in example 2, further using High Resolution Melt analysis or alternatively by gel electrophoresis.

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