Title: A METHOD OF DETERMINING THE SEX OF INDONESIAN RED AROWANAS

Abstract: The present invention relates to a method of determining the sex of Indonesian arowanas. Also, the present invention relates to a kit for determining the sex of Indonesian red arowanas and a method of using thereof.

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A METHOD OF DETERMINING THE SEX OF INDONESIAN RED AROWANAS

FIELD OF THE TECHNOLOGY

The field of the present technology relates to a method of determining the sex of Indonesian red arowanas. Additionally, the field of the present technology relates to a kit for determining the sex of Indonesian red arowanas and a method of using thereof.

BACKGROUND

Over the past few decades, the Asian arowana Scleropages formosus of the Osteoglossidae family has acquired a special status as a very popular but extremely expensive aquarium or ornamental fish. Asian arowanas, commonly known as dragon fish, are generally found in three main colour varieties: red, golden, and green. Red arowanas are among the most valued owing in part to their resemblance to the Chinese dragon and positive Feng Shui associations with water and the colour red. The market price for red arowanas can be much higher than that of other arowanas; for example, red arowanas can cost over USD $4,000.

The special status of the Asian arowana, including the red variety, in the aquarium or ornamental fish industry has led to overfishing. Overfishing has subsequently resulted in the Asian arowana being listed as an endangered species by the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). Therefore, the trade of Asian arowanas is generally prohibited in member countries of CITES unless the Asian arowanas are bred in captivity.

However, captive breeding for conservation and production purposes has to date presented unsolved challenges. Specifically, the optimal sex ratio of breeding populations is difficult to establish. For example, the gender of Asian arowanas can typically only be visually ascertained after the Asian arowanas reach sexual maturity at 3-4 years of age when the fish begin to breed and the males carry eggs in their mouths.
Other methods for sexing Asian arowanas do exist. For example, the Arowana Gender Index (AGI) uses the size of the fish's head to determine gender. However, the fish must be at least 1 year old, the method is not applicable to deformed or unhealthy fish, and the method has an accuracy of only 70%. The gross anatomy method includes dissection of the fish including the gonad and subsequent visual inspection of the gonad for sex determination, while the histological analysis method utilizes a microscope to study the dissected gonad for sex determination. In both the gross anatomy and histological analysis methods, however, the fish being sexed dies resulting in obvious drawbacks to breeding efforts. Further, the visual methods of determining the sex of Asian arowanas can potentially be unreliable due to human error and the like.

Molecular sexing in fish is much more difficult than in mammals and, so far, no single universal sex-associated DNA marker has been identified for any fish species, presumably due to the complex or diverse nature of sex determination systems in fish. A previous attempt at using a sex-specific DNA marker for sexing green arowanas resulted in a method of sexing green arowanas with an accuracy of 82.7%. Green arowanas, however, are the most common variety of the Asian arowana and are relatively widespread throughout Southeast Asia. On the other hand, red arowanas are considered to be highly endangered and highly valued; thus, there is a need to develop a convenient and accurate sexing method and kit that can be used with red arowanas to establish the optimal sex ratio of breeding populations to be employed during captive breeding for conservation and production purposes.

SUMMARY

A first aspect provides a method of determining the sex of Indonesian red arowanas including: providing one or more Indonesian red arowanas; isolating a genomic DNA sample from the one or more Indonesian red arowanas; and identifying a sex-specific DNA marker for Indonesian red arowanas by using Polymerase Chain Reaction (hereinafter "PCR") with a first primer and second primer. In embodiments, the identifying step comprises amplifying the sex-specific DNA marker.
In embodiments, the method of determining the sex of Indonesian red arowanas can have an accuracy of at least 83% for sexing the one or more Indonesian red arowanas using the sex-specific DNA marker. In embodiments, the method of determining the sex of Indonesian red arowanas can have an accuracy of at least 90% for sexing the one or more Indonesian red arowanas using the sex-specific DNA marker. In embodiments, the method of determining the sex of Indonesian red arowanas can have an accuracy of at least 95% for sexing the one or more Indonesian red arowanas using the sex-specific DNA marker. In embodiments, the method of determining the sex of Indonesian red arowanas can have an accuracy of about 100% for sexing the one or more Indonesian red arowanas using the sex-specific DNA marker.

In embodiments, the sex-specific DNA marker for Indonesian red arowanas is a female-specific DNA marker. In embodiments, the female-specific DNA marker includes 320 nucleotides. In embodiments, the female-specific DNA marker is at least 98% homologous, at least 99% homologous, or 100% homologous with SEQ. ID. 1 (shown in FIG 1).

In embodiments, the first primer has an oligonucleotide sequence of 5' TAACTCAAAAGTAGAATAGAACAATG 3'. In embodiments, the second primer has an oligonucleotide sequence of 5' AATTCAAGGGAACTGATGACTCTA 3'.

In embodiments, the one or more genomic DNA samples can be isolated from a DNA source, such as but not limited to, muscle, gill, fin, mucus, stool or a combination of one or more thereof. In embodiments, the one or more genomic DNA samples can be isolated from stool.
In embodiments, the identifying of the sex-specific DNA marker further includes the use of a PCR reaction mix and the use of PCR cycling conditions. In embodiments, the PCR reaction mix includes: 0.5 µ l of 10 µM solution of the first primer; 0.5 µ l of 10 µM solution of the second primer; 1.25 µ l of 10X PCR ImmoBuffer; 0.375 µ l of 50 mM MgCl₂; 0.25 µ l of 10 mM dNTP (deoxyribonucleotide triphosphate); 8.075 µ l of double-distilled water; 0.05 µ l of 5 U/µ l IMMOLASE™ DNA Polymerase (Bioline, UK); and 3 µ l of 25 ng/µ l isolated genomic DNA.

In embodiments, the PCR cycling conditions include: an initial denaturation at 95 °C for 10 minutes; 35 cycles of denaturation at 95 °C for 30 seconds, annealing at an optimal annealing temperature of 59 °C for 30 seconds and extension at 72 °C for 30 seconds; a final extension step at 72 °C for 10 minutes; and a hold step at 4 °C.

In embodiments, the method of determining the sex of Indonesian red arowanas can further include identifying a species-associated DNA marker for Indonesian red arowanas by using Polymerase Chain Reaction with a third primer and fourth primer, wherein the third primer has an oligonucleotide sequence of 5' ACACAGTGGGACAGGGAATCT3', wherein the fourth primer has an oligonucleotide sequence of 5' GAAACCAGAGTTGGGACAGGTC3', wherein the identifying of the species-associated DNA marker includes amplifying the species-associated DNA marker, and wherein the species-associated DNA marker is at least 98% homologous, at least 99% homologous, or 100% homologous with SEQ ID. 2 (shown in FIG 2).

In embodiments, the identifying of the sex-specific DNA marker and the identifying of the species-associated DNA marker includes the use of a PCR reaction mix and the use of PCR cycling conditions. In embodiments, the PCR reaction mix includes: 0.5 µ l of 10 µM of the first primer; 0.5 µ l of 10 µM of the second primer; 0.25 µ l of 10 µM of the third primer; 0.25 µ l of 10 µM of the fourth primer; 1.25 µ l of 10X PCR ImmoBuffer; 0.375 µ l of 50 mM MgCl₂; 0.25 µ l of 10 mM dNTP (deoxyribonucleotide triphosphate); 7.575 µ l of double-distilled water; 0.05 µ l of 5 U/µ l IMMOLASE™ DNA Polymerase (Bioline, UK); and 3 µ l of
25 ng/µl isolated genomic DNA. In embodiments, the PCR cycling conditions include: an initial denaturation at about 95 °C for about 10 minutes; about 32 cycles of denaturation at about 95 °C for about 30 seconds, annealing at an optimal annealing temperature of about 61 °C for about 30 seconds and extension at about 72 °C for about 30 seconds; a final extension step at about 72 °C for about 10 minutes; and a hold step at about 4 °C.

In embodiments, the method of determining the sex of Indonesian red arowanas can further include confirming that the one or more isolated genomic DNA samples are from Indonesian red arowanas rather than from contaminant DNA from microorganisms, contaminant DNA from animals used as fish feed or a combination thereof.

A second aspect provides a kit for use in determining the sex of one or more Indonesian red arowanas including: a first primer having an oligonucleotide sequence of 5’ TAACTCAAAGTAGAATAGAACAATG3’, a second primer having an oligonucleotide sequence of 5’ AATTCAAGGGAACTGACTCTA 3’, and IMMOLASE™ DNA Polymerase (Bioline, UK).

A third aspect provides a kit for determining the species and sex of one or more Indonesian red arowanas in one step or simultaneously including: a first primer having an oligonucleotide sequence of 5’ TAACTCAAAGTAGAATAGAACAATG3’, a second primer having an oligonucleotide sequence of 5’ AATTCAAGGGAACTGACTCTA 3’, a third primer having an oligonucleotide sequence of 5’ ACACAGTGCAAGGTAACAGATCT 3’, a fourth primer having an oligonucleotide sequence of 5’ GAAACCAGAGTGAGGGACAGGTC 3’, and IMMOLASE™ DNA Polymerase (Bioline, UK).

**BRIEF DESCRIPTION OF THE DRAWING**

Embodiments of the present disclosure are described herein with reference to the drawings in which:
FIG. 1 illustrates the 97% homology between the sequence of the sex-specific DNA marker band (GenBank Accession No. AF391095.1) for Asian green arowanas and the sequence of the 320 bp (base pair) DNA band discovered by the inventors to be the sex-specific DNA marker of female Indonesian red arowanas.

FIG. 2 depicts the sequence of the 125 bp (base pair) DNA band that can be used as a species-associated marker of Asian arowanas including Indonesian red arowanas.

FIG. 3 illustrates a multiplex PCR kit for species and sex determination of Indonesian red arowanas in one step or simultaneously.

FIG. 4 illustrates the use of a pair of primers DSX66A and DSX66B, a specific PCR reaction mix, and specific PCR cycling conditions to amplify a 320 bp DNA band found in female Indonesian red arowanas.

FIG. 5 illustrates the use of two pairs of PCR primers DSX66A/DSX66B and DSX06A/DSX06B, a specific PCR reaction mix, and specific PCR cycling conditions to amplify a 125 bp DNA band found in Indonesian red arowanas and to amplify a 320 bp DNA band found in female Indonesian red arowanas.

FIG. 6 illustrates the use of a pair of primers Arol-F and DSX06B, a specific PCR reaction mix, and specific PCR cycling conditions to amplify a 900 bp DNA band found exclusively in female Asian arowanas.

DETAILED DESCRIPTION

In the following detailed description, reference is made to the accompanying drawings, which form a part hereof. In the drawings, similar symbols typically identify similar components, unless context dictates otherwise. The illustrative embodiments described in the detailed description, drawings, and claims are not meant to be limiting. Other
embodiments can be utilized, and other changes can be made, without departing from the spirit or scope of the subject matter presented herein.

Unless specified otherwise, the terms "comprising" and "comprise" as used herein, and grammatical variants thereof, are intended to represent "open" or "inclusive" language such that they include recited elements but also permit inclusion of additional, un-recited elements.

As used herein, the term "about", in the context of concentrations of components, conditions, other measurement values, etc., means +/- 5% of the stated value, or +/- 4% of the stated value, or +/- 3% of the stated value, or +/- 2% of the stated value, or +/- 1% of the stated value, or +/- 0.5% of the stated value, or +/- 0% of the stated value.

Throughout this disclosure, certain embodiments may be disclosed in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the disclosed ranges. Accordingly, the description of a range should be considered to have specifically disclosed all the possible sub-ranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed sub-ranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 3, 4, 5, and 6. This applies regardless of the breadth of the range.

As used herein, the term "Asian arowanas" can include Asian red arowanas, Asian golden arowanas, Asian green arowanas and Asian black arowanas.

Isolation of Genomic DNA Samples

In embodiments, for the identification of a sex-specific DNA marker and/or a species-associated DNA marker in Indonesian red arowanas, the phenol-chloroform method or
similar method known in the art can be used to extract and isolate genomic DNA samples from muscles, gills, scales, fins and/or mucus of male and female Indonesian red arowanas.

In embodiments, genomic DNA samples can be extracted and isolated from excretory products (i.e., stool and/or mucus) of male and female Indonesian red arowanas in an aquarium or the like. In embodiments, a QIAamp DNA Stool Mini Kit (QIAGEN, Hilden, Germany) or similar kit known in the art can be used to extract and isolate genomic DNA samples from excretory products.

The present disclosure relates to a genomic DNA collection kit that can be used for collecting excreted products, such as, stool and/or mucus, Indonesian-red-arowanas and other Asian arowanas. In embodiments, the genomic DNA collection kit can include equipment for collecting stool and/or mucus. In embodiments, the equipment can include a sterilization roll that can be used to sieve stool and/or mucus from water. In embodiments, the sieved mucus and stool can be attached to a surface of fiberglass in a container or box. The fiberglass with the attached stool and/or mucus can be removed from the container or box for testing purposes.

In embodiments, the equipment can also include swabs for collecting stool and/or mucus directly from Indonesian red arowanas and other Asian arowanas. In embodiments, the part of the swab that is used to directly touch the body of the fish to collect the stool and/or mucus can be made of rayon fiber.

**Quantification and Qualitative Analysis of the Isolated Genomic DNA samples**

In embodiments, a spectrophotometric method can be used to quantify and qualitatively analyze the isolated genomic DNA samples. In embodiments, a GeneQuant RNA/DNA Calculator Spectrophotometer from Amersham or similar spectrophotometer known in the art can be used to quantify and qualitatively analyze the isolated DNA samples.
The spectrophotometer can be used to determine the optical density of the isolated genomic DNA samples at 260 nm and 280 nm. The concentration and/or volume of the isolated genomic DNA samples can be determined by measuring the optical density at 260 nm (i.e., \( \text{OD}_{260} \)). The peak absorption of DNA is at 260 nm and there is a simple relationship between the amount of light absorbed and the concentration of the isolated genomic DNA sample:

\[
1 \text{ OD}_{260} \text{ unit} = 50 \text{ mg/ml DNA}
\]

The purity of the isolated genomic DNA samples can be determined by measuring the optical density at 280 nm (i.e., \( \text{OD}_{280} \)) and calculating the ratio of the optical density reading at 260 nm to the optical density reading at 280 nm (i.e., \( \text{OD}_{260} / \text{OD}_{280} \)). In general, DNA absorbs almost twice as much light at 260 nm than at 280 nm. A ratio of 1.8 or better can indicate that the isolated genomic DNA sample is sufficiently pure. A ratio lower than 1.8 can indicate that there is a contaminant in the isolated genomic DNA sample that is absorbing 280 nm light (i.e., a protein contaminant). A ratio significantly greater than 1.8 can indicate that some DNA degradation or RNA contamination may have occurred.

In embodiments, an electrophoresis method can be used to quantify and qualitatively analyse the isolated genomic DNA samples. In embodiments, a Thermo E-C Minicell Primo EC320 electrophoresis chamber or similar electrophoresis instrument known in the art can be used to quantify and qualitatively analyse the isolated genomic DNA samples.

**Sex Determination using a Polymerase Chain Reaction (PCR) Method and PCR Primer**

**Pair of PSX66A and DSX66B**

The present disclosure relates to a method of determining the sex of Indonesian red arowanas. In embodiments, female Indonesian red arowanas can be differentiated from male Indonesian red arowanas by using PCR primers DSX66A and DSX66B designed by YUE, et al. The pair of PCR primers have the following oligonucleotide sequences:

DSX66A: 5' TAACTCAAAAGTAGAATAGAACAATG 3'
DSX66B: 5' AATTC A A G G G A ACTG ATG ACTCTA 3'

In embodiments, the quantity of an isolated genomic DNA sample can be increased via a PCR method including the use of a specific PCR reaction mix (including the pair of PCR primers) and specific PCR cycling conditions.

**PCR Reaction Mix for Sex Determination**

In embodiments, female Indonesian red arowana can be differentiated from male Indonesian red arowanas by using a PCR reaction mix having a volume of 14 μl that can include: about 0.5 μl of 10 μM DSX66A (PCR primer); about 0.5 μl of 10 μM DSX66B (PCR primer); about 1.25 μl of 10X PCR ImmoBuffer; about 0.375 μl of 50 mM MgCl2; about 0.25 μl of 10 mM dNTP (deoxyribonucleotide triphosphate); about 8.075 μl of ddH20 (double-distilled water); about 0.05 μl of 5 U/μl IMMOLASE™ DNA Polymerase (Bioline, UK); and about 3 μl of 25 ng/μl isolated genomic DNA.

**PCR Cycling Conditions for Sex Determination**

In embodiments, female Indonesian red arowanas can be differentiated from male Indonesian red arowanas by using PCR cycling conditions that can include: an initial denaturation at about 95 °C for about 10 minutes; about 35 cycles of denaturation at about 95 °C for about 30 seconds, annealing at an optimal annealing temperature of about 59 °C for about 30 seconds and extension at about 72 °C for about 30 seconds; a final extension step at about 72 °C for about 10 minutes; and a hold step at about 4 °C for an indefinite period of time.

In embodiments, PCR was achieved using a temperature-gradient PCR machine (Veriti® Thermal Cycler, Applied Biosystems®, USA). The PCR products were separate and analysed using agarose gel electrophoresis. The PCR products were separated on 1.5% agarose gel.
In embodiments, the specific PCR reaction mix (including the quantity of each component and the brand of each component) described above and the specific PCR cycling conditions described above can be used to amplify a 320 bp DNA band found in female Indonesian red arowanas. Therefore, the 320 bp DNA band can be used as a sex-specific DNA marker to identify the sex of female Indonesian red arowanas and male Indonesian red arowanas. For example, if a 320 bp DNA band is amplified in an Indonesian red arowana, the particular Indonesian red arowana is a female. On the other hand, if a 320 bp DNA band is not amplified in an Indonesian red arowana, the particular Indonesian red arowana is a male.

YUE, et al. designed the pair of PCR primers DSX66A AND DSX66B based on the sex-specific DNA marker (GenBank Accession No. AF391095.1) for _Arowanas._ As shown in FIG. 1, there is a 97% homology between the sequence of the specific DNA marker (AF391095.1) for green arowanas and the sequence of the 320 bp DNA band discovered by the inventors to be the sex-specific marker of Indonesian red arowanas. As such, the inventors were able to utilize the pair of PCR primers DSX66A AND DSX66B to amplify and identify the 320 bp DNA band, the sex-specific DNA marker for Indonesian red arowanas. It is known in the art that a 268 bp DNA band is the sex-specific DNA marker for Asian green arowanas.

In embodiments, a method of determining the sex of Indonesian red arowanas can include the use of the specific PCR reaction mix (including the quantity of each component and the brand of each component) described above and the specific PCR cycling conditions described above, wherein a 320 bp DNA band is amplified in female Indonesian red arowanas, wherein the 320 bp DNA band can be used as a sex-specific DNA marker to identify the sex of female Indonesian red arowanas and male Indonesian red arowanas. In embodiments, the method of determining the sex of Indonesian red arowanas can have an accuracy of at least 83%, at least 85%, at least 87%, at least 89%, at least 91%, at least 93%, at least 95%, at least 97%, at least 99%, or 100% for sexing the
one or more Indonesian red arowanas using the 320 bp DNA band as a sex-specific DNA marker.

Surprisingly and unexpectedly, the inventors discovered that the use of the specific PCR reaction mix (including the quantity of each component and the brand of each component) described above and the specific PCR cycling conditions described above in a method of determining the sex of Indonesian red arowanas resulted in the method having an accuracy of 100% for sexing the one or more Indonesian red arowanas. Surprisingly and unexpectedly, the inventors discovered that the PCR reaction mix and PCR cycling conditions could be adjusted to develop a method of determining the sex of Indonesian red arowanas having an accuracy of 100%. In particular, the inventors discovered that the quantity of DNA in the PCR reaction mix, the enzyme brand in the PCR reaction mix, and the PCR cycling conditions including the optimal annealing temperature critically affected the accuracy of the method of molecular sexing of the present disclosure.

Molecular sexing of the highly valued and highly endangered Indonesian red arowana has not been successfully conducted until now. Therefore, the result of developing a method and kit for molecular sexing of Indonesian red arowanas with 100% accuracy is surprising and unexpected. The method of molecular sexing of the present disclosure can be utilized to establish the optimal sex ratio of Indonesian red arowana breeding populations to be employed during captive breeding of Indonesian red arowanas for conservation and production purposes.

In embodiments, the present disclosure relates to a PCR kit for determining the sex of Indonesian red arowanas, wherein the PCR kit includes the PCR reaction mix components described above.
Species Determination or Identification using Polymerase Chain Reaction (PCR) Primers

The present disclosure relates to a method of species determination of Indonesian red arowanas. In embodiments, genomic DNA samples can be isolated from the feces or stool of Indonesian red arowanas. In embodiments, the genomic DNA collection kit of the present disclosure or a similar kit can be used to isolate the feces or stool of Indonesian red arowanas. In embodiments, PCR primers DSX06A and DSX06B designed by YUE, et al. can be used to identify a species-associated DNA marker and thereby confirm that the DNA samples are DNA from Indonesian red arowana rather than contaminant DNA from microorganisms and/or contaminant DNA from animals used as fish feed. The pair of PCR primers have the following sequences:

DSX06A: 5' ACACAGTGCAAGTAACAGAATCT 3'
DSX06B: 5' GAAACCAGAGTTGGGACAGGTC 3'

In embodiments, the quantity of an isolated genomic DNA sample can be increased via a PCR method including the use of a PCR reaction mix (including the pair of PCR primers) and specific PCR cycling conditions.

PCR Reaction Mix for Species Determination or Identification

In embodiments, genomic DNA from Indonesian red arowanas can be differentiated from contaminant DNA from microorganisms and/or contaminant DNA from animals used as fish feed by using a PCR reaction mix having a volume of about 14 µl that can include:
- about 0.5 µl of 10 µM DSX06A (PCR primer);
- about 0.5 µl of 10 µM DSX06B (PCR primer);
- about 1.25 µl of 10X ImmoBuffer;
- about 0.375 µl of 50 mM MgCl₂;
- about 0.25 µl of 10 mM dNTP (deoxyribonucleotide triphosphate);
- about 8.075 µl of ddH₂O (double-distilled water);
- about 0.05 µl of 5 U/µl IMMOLASE™ DNA Polymerase (Bioline, UK); and about 3 µl of a 25 ng/µl isolated genomic DNA.

PCR Cycling Conditions for Species Determination or Identification

In embodiments, genomic DNA from Indonesian red arowanas can be differentiated from contaminant DNA from microorganisms and/or contaminant DNA from animals used as
fish feed by using PCR cycling conditions that can include: an initial denaturation at about
95 °C for about 10 minutes; about 35 cycles of denaturation at about 95 °C for about 30
seconds, annealing at an optimal annealing temperature of about 55 °C for about 30
seconds and extension at about 72 °C for about 30 seconds; a final extension step at
about 72 °C for about 10 minutes; and a hold step at about 4 °C for an indefinite period of
time.

In embodiments, PCR was achieved using a temperature-gradient PCR machine (Veriti®
Thermal Cycler, Applied Biosystems®, USA). The PCR products were separate and
analysed using agarose gel electrophoresis. The PCR products were separated on 1.5%
agarose gel.

In embodiments, the PCR reaction mix and the PCR cycling conditions can be used to
amplify a 125 bp DNA band found in Indonesian red arowanas. Therefore, the 125 bp
DNA band can be used as a species-associated DNA marker to differentiate between DNA
of the Indonesian red arowanas and the contaminant DNA of microorganisms and/or
contaminant DNA of animals used as fish feed. For example, if a 125 bp DNA band is
amplified in a DNA sample isolated from feces or stool, this particular DNA sample is
derived from an Indonesian red arowana. On the other hand, if a 125 bp DNA band is not
amplified in the DNA sample isolated from feces or stool, this particular DNA sample is
derived from a microorganism or animal used as fish feed.

The present disclosure also relates to a method of species determination of Asian
arowanas. In embodiments, genomic DNA samples can be isolated from the feces
or stool of Asian arowanas. In embodiments, the genomic DNA collection kit of the
present disclosure or a similar kit can be used to isolate the feces or stool of Asian
arowanas. In embodiments, PCR primers DSX06A and DSX06B designed by YUE, et
al. can be used to identify a species-associated DNA marker and thereby confirm
that the DNA samples are DNA from Asian arowana rather than contaminant DNA
from microorganisms and/or contaminant DNA from animals used as fish feed. The pair of PCR primers have the following sequences:

DSX06A: 5' ACACAGTGCAGGTAACAGAATCT 3'
DSX06B: 5' GAAACCAGAGTTGGGACAGGTC 3'

In embodiments, the quantity of an isolated genomic DNA sample can be increased via a PCR method including the use of a PCR reaction mix (including the pair of PCR primers) and specific PCR cycling conditions.

In embodiments, genomic DNA from Asian arowanas can be differentiated from contaminant DNA from microorganisms and/or contaminant DNA from animals used as fish feed by using a PCR reaction mix having a volume of about 30 µl that can include: about 0.5 µl of 10 µM DSX06A (PCR primer); about 0.5 µl of 10 µM DSX06B (PCR primer); about 1.25 µl of 10X ImmoBuffer; about 0.375 µl of 50 mM MgCl2; about 0.25 µl of 10 mM dNTP (deoxyribonucleotide triphosphate); about 8.075 µl of ddH2O (double-distilled water); about 0.05 µl of 5 U/µl IMMOLASE™ DNA Polymerase (Bioline, UK); and about 3 µl of a 25 ng/µl isolated genomic DNA.

In embodiments, genomic DNA from Asian arowanas can be differentiated from contaminant DNA from microorganisms and/or contaminant DNA from animals used as fish feed by using PCR cycling conditions that can include: an initial denaturation at about 95 ºC for about 10 minutes; about 35 cycles of denaturation at about 95 ºC for about 30 seconds, annealing at an optimal annealing temperature of about 55 ºC for about 30 seconds and extension at about 72 ºC for about 30 seconds; a final extension step at about 72 ºC for about 10 minutes; and a hold step at about 4 ºC for an indefinite period of time.

In embodiments, PCR was achieved using a temperature-gradient PCR machine (Veriti ® Thermal Cycler, Applied Biosystems ® , USA). The PCR products were
separate and analysed using agarose gel electrophoresis. The PCR products were separated on 1.5% agarose gel.

As illustrated in FIG. 2, in embodiments, the PCR reaction mix and the PCR cycling conditions can be used to amplify a 125 bp DNA band found in Asian arowanas. Therefore, the 125 bp DNA band can be used as a species-associated DNA marker to differentiate between DNA of the Asian arowanas and the contaminant DNA of microorganisms and/or contaminant DNA of animals used as fish feed. For example, if a 125 bp DNA band is amplified in a DNA sample isolated from feces or stool, this particular DNA sample is derived from an Asian arowana. On the other hand, if a 125 bp DNA band is not amplified in the DNA sample isolated from feces or stool, this particular DNA sample is derived from a microorganism or animal used as fish feed.

Sex Determination using a Polymerase Chain Reaction (PCR) Method and PCR Primer Pair of Arol-F and DSX06B

In embodiments, female Asian arowanas (i.e., Indonesian red arowanas, Malaysian gold arowanas, Asian green arowanas, and Asian black arowanas) can be differentiated from male Asian arowanas (i.e., Indonesian red arowanas, Malaysian gold arowanas, Asian green arowanas, and Asian black arowanas) by using the PCR primer pair of Arol-F (designed by the inventors of the present disclosure) and DSX06B (designed by YUE, et al). The pair of PCR primers have the following sequences:

Arol-F: 5' AGGACCGGAAGGAGTGGGC 3'

DSX06B: 5' GAAACCAGAGGTGGGACAGGTC 3'

The inventors designed Arol-F using a mitochondrial genome sequence of arowana found in the NCBI database.
In embodiments, the quantity of an isolated genomic DNA sample can be increased via a PCR method including the use of a PCR reaction mix (including the pair of PCR primers) and specific PCR cycling conditions.

**PCR Reaction Mix for Sex Determination (using PCR Primer Pair of Arol-F and DSX06B)**

In embodiments, female Asian arowanas (i.e., Indonesian red arowanas, Malaysian gold arowanas, Asian green arowanas, and Asian black arowanas) can be differentiated from male Asian arowanas (i.e., Indonesian red arowanas, Malaysian gold arowanas, Asian green arowanas, and Asian black arowanas) by using a PCR reaction mix having a volume of about 25 μl that can include: about 0.5 μl of 10 μM Arol-F (PCR primer); about 0.5 μl of 10 μM DSX06B (PCR primer); about 2.5 μl of 10X PCR Buffer with 15 mM MgCl₂; about 2.00 μl of 1.25 mM dNTP (deoxyribonucleotide triphosphate); about 17.20 μl of ddH₂O (double-distilled water); about 0.30 μl of 5 U/μl RBC Taq DNA Polymerase; and about 2 μl of 25 ng/μl isolated genomic DNA.

**PCR Cycling Conditions for Sex Determination (using PCR Primer Pair of Arol-F and DSX06B)**

In embodiments, female Asian arowanas (i.e., Indonesian red arowanas, Malaysian gold arowanas, Asian green arowanas, and Asian black arowanas) can be differentiated from male Asian arowanas (i.e., Indonesian red arowanas, Malaysian gold arowanas, Asian green arowanas, and Asian black arowanas) by using PCR cycling conditions that can include: an initial denaturation at about 95 °C for about 11 minutes; about 35 cycles of denaturation at about 94 °C for about 1 minute, annealing at an optimal annealing temperature of about 54 °C for about 1 minute and extension at about 72 °C for about 1 minute; a final extension step at about 72 °C for about 10 minutes; and a hold step at about 4 °C for an indefinite period of time.
In embodiments, PCR was achieved using a temperature-gradient PCR machine (Veriti® Thermal Cycler, Applied Biosystems®, USA). The PCR products were separate and analysed using agarose gel electrophoresis. The PCR products were separated on 1.5% agarose gel.

In embodiments, the specific PCR reaction mix and the specific PCR cycling conditions can be used to amplify a 900 bp DNA band found exclusively in female Asian arowanas (i.e., Indonesian red arowanas, Malaysian gold arowanas, Asian green arowanas, and Asian black arowanas). Therefore, the 900 bp DNA band can be used as a sex-specific DNA marker to identify the sex of female Asian arowanas and male Asian arowanas. For example, if a 900 bp DNA band is amplified in an Asian arowana (i.e., Indonesian red arowana, Malaysian gold arowana, Asian green arowana, and Asian black arowana), this particular Asian arowana is a female. On the other hand, if a 900 bp DNA band is not amplified in an Asian arowana (i.e., Indonesian red arowana, Malaysian gold arowana, Asian green arowana, and Asian black arowana), this particular Asian arowana is a male.

Multiplex PCR with Two Pairs of PCR Primers DSX06A/DSX06B and DSX66A/DSX66B for Species and Sex Determination in One Step or Simultaneously

The present disclosure relates to a method of determining the species and sex of Indonesian red arowanas in one step or simultaneously. In embodiments, two pairs of PCR primers DSX06A/DSX06B and DSX66A/DSX66B can be used for determining the species and sex of Indonesian red arowanas in one step or simultaneously.

In embodiments, female Indonesian red arowanas can be differentiated from male Indonesian red arowanas by using PCR primers DSX66A and DSX66B designed by YUE, et al. The pair of PCR primers have the following sequences:

DSX66A: 5' TAACTCAAAAGTAGAAATAGAAATG 3'
DSX66B: 5' AATTCAAGGAACTGATGACTCTA 3'
At the same time, the PCR primer pair of DSX06A and DSX06B designed by YUE, et al. can be used to identify a species-associated DNA marker and, thereby, confirm that the DNA samples are DNA from Indonesian red arowana rather than contaminant DNA from microorganisms and/or contaminant DNA from animals used as fish feed. The pair of PCR primers have the following sequences:

DSX06A: 5' ACACAGTGCAAGTAACGAATCT 3'
DSX06B: 5' GAAACCAGAGTTGGGACAGGTC 3'

In embodiments, the quantity of an isolated genomic DNA sample can be increased via a PCR method including the use of a PCR reaction mix (including the two pairs of PCR primers) and specific PCR cycling conditions.

**Multiplex PCR Reaction Mix**

In embodiments, the multiplex PCR reaction mix for species and sex determination of Indonesian red arowanas in one step or simultaneously include: about 0.5 µl of about 10 µM DSX66A; about 0.5 µl of about 10 µM DSX66B; about 0.25 µl of about 10 µM DSX06A; about 0.25 µl of about 10 µM DSX06B; about 1.25 µl of about 10X PCR ImmoBuffer; about 0.375 µl of about 50 mM MgCl₂; about 0.25 µl of about 10 mM dNTP (deoxyribonucleotide triphosphate); about 7.575 µl of double-distilled water; about 0.05 µl of about 5 U/µl IMMOLASE™ DNA Polymerase (Bioline, UK); and about 3 µl of about 25 ng/µl isolated genomic DNA.

**Multiplex PCR Cycling Conditions**

In embodiments, the multiplex PCR cycling conditions for species and sex determination of Indonesian red arowanas in one step or simultaneously include: an initial denaturation at about 95 °C for about 10 minutes; about 32 cycles of denaturation at about 95 °C for about 30 seconds, annealing at an optimal annealing temperature of about 61 °C for about 30 seconds and extension at about 72 °C for about 30 seconds; a final extension step at about 72 °C for about 10 minutes; and a hold step at about 4 °C.
In embodiments, PCR was achieved using a temperature-gradient PCR machine (Veriti® Thermal Cycler, Applied Biosystems®, USA). The PCR products were separate and analysed using agarose gel electrophoresis. The PCR products were separated on 1.5% agarose gel.

In embodiments, the specific multiplex PCR reaction mix (including the quantity of each component and the brand of each component) described above and the specific multiplex PCR cycling conditions described above can be used to amplify a 320 bp DNA band found in female Indonesian red arowanas. Therefore, the 320 bp DNA band can be used as a sex-specific DNA marker to identify the sex of female Indonesian red arowanas and male Indonesian red arowanas. For example, if a 320 bp DNA band is amplified in an Indonesian red arowana, this particular Indonesian red arowana is a female. On the other hand, if a 320 bp DNA band is not amplified in an Indonesian red arowana, this particular Indonesian red arowana is a male.

The 320 bp DNA band is identified via the use of the pair of PCR primers DSX66A/DSX66B.

In embodiments, a method of determining the species and sex of Indonesian red arowanas in one step or simultaneously can include the use of the specific multiplex PCR reaction mix (including the quantity of each component and the brand of each component) described above and the specific multiplex PCR cycling conditions described above, wherein a 320 bp DNA band is amplified in female Indonesian red arowanas, wherein the 320 bp DNA band can be used as a sex-specific DNA marker to identify the sex of female Indonesian red arowanas and male Indonesian red arowanas. In embodiments, the method of determining the species and sex of Indonesian red arowanas in one step or simultaneously can have an accuracy of at least 83%, at least 85%, at least 87%, at least 89%, at least 91%, at least 93%, at least 95%, at least 97%, at least 99%, or 100% for determining the sex of the one or more Indonesian red arowanas using the 320 bp DNA band as a sex-specific DNA marker.
Surprisingly and unexpectedly, the inventors discovered that the use of the specific multiplex PCR reaction mix (including the quantity of each component and the brand of each component) described above and the specific multiplex PCR cycling conditions described above in a method of determining the species and sex of Indonesian red arowanas in one step or simultaneously resulted in the method having an accuracy of 100% for sexing the one or more Indonesian red arowanas. Surprisingly and unexpectedly, the inventors discovered that the multiplex PCR reaction mix and multiplex PCR cycling conditions could be adjusted to develop a method of determining the sex of one or more Indonesian red arowanas having an accuracy of 100%. In particular, the inventors discovered that the quantity of DNA in the PCR reaction mix, the enzyme brand in the PCR reaction mix, and the PCR cycling conditions including the optimal annealing temperature affect the accuracy of determining the sex of Indonesian red arowanas.

Molecular sexing of the highly valued and highly endangered Indonesian red arowana has not been successfully conducted until now. Therefore, the result of developing a method of molecular sexing of Indonesian red arowanas with 100% accuracy is surprising and unexpected. The method of molecular sexing of the present disclosure can be utilized to establish the optimal sex ratio of Indonesian red arowana breeding populations to be employed during captive breeding of Indonesian red arowanas for conservation and production purposes.

In embodiments, the multiplex PCR reaction mix and the multiplex PCR cycling conditions can be used to amplify a 125 bp DNA band found in Indonesian red arowanas. Therefore, the 125 bp DNA band can be used as a species-associated DNA marker to differentiate between DNA of the Indonesian red arowanas and the contaminant DNA of microorganisms and/or contaminant DNA of animals used as fish feed. For example, if a 125 bp DNA band is amplified in a DNA sample isolated from feces or stool, this particular DNA sample is derived from an Indonesian red arowana. On the other hand, if a 125 bp DNA band is not amplified in the DNA
sample isolated from feces or stool, this particular DNA sample is derived from a microorganism or animal used as fish feed. The 125 bp DNA band is identified via the use of the pair of PCR primers DSX06A/DSX06B. In embodiments, the method of determining the species and sex of Indonesian red arowanas in one step or simultaneously can have an accuracy of about 100% for determining the species of the one or more Indonesian red arowanas using the 125 bp DNA band as a species-associated DNA marker.

Multiplex PCR Kit for Species and Sex Determination in One Step or Simultaneously

Using the method of determining the species and sex of Indonesian red arowanas in one step or simultaneously described above, a multiplex PCR kit for species and sex determination in one step or simultaneously was developed. Accordingly, the present disclosure also relates to a multiplex PCR kit for determining the species and sex of Indonesian red arowanas in one step or simultaneously. The present disclosure further relates to a method of using the multiplex PCR Kit for determining the species and sex of Indonesian red arowanas in one step or simultaneously. In embodiments, the multiplex PCR kit includes the multiplex PCR reaction mix components described above. In embodiments, the method of using the multiplex PCR kit includes the multiplex PCR cycling conditions described above.

The multiplex PCR kit for determining the species and sex of Indonesian red arowanas and the method of using thereof provide for a more convenient and faster method of determining both the species and sex of Indonesian red arowanas in one step or simultaneously. The one step multiplex PCR kit and method of using thereof results in a decrease in the amount of PCR reaction mix components (i.e., PCR primers, IMMOLASE™ DNA Polymerase (Bioline, UK), dNTP, PCR ImmoBuffer, MgCl₂, double-distilled water, genomic DNA sample) required, a decrease in the amount of time required to conduct both species and sex determination, and a decrease in testing costs.
Referring to FIG. 3, in embodiments, the multiplex PCR kit including the cover package 10 for determining the species and sex of Indonesian red arowanas in one step or simultaneously can include: a 10.95 µl Species and Sex Master Mix 12, which can include 0.5 µl of 10 µM DSX66A, 0.5 µl of 10 µM DSX66B, 0.25 µl of 10 µM DSX06A, 0.25 µl of 10 µM DSX06B, 1.25 µl of 10X PCR ImmoBuffer, 0.375 µl of 50 mM MgCl₂, 0.25 µl of 10 mM dNTP (deoxyribonucleotide triphosphate), and 7.575 µl of double-distilled water. The multiplex PCR kit can also include: 0.05 µl 5 U/µl IMMOLASE™ DNA Polymerase (Bioline, UK) 14. The multiplex PCR kit can also include a Species and Sex positive control 16, which includes 3 µl of 25 ng/ml genomic DNA of a female Indonesian red arowana. The multiplex PCR kit can also include a negative control 18, which includes double-distilled water. The multiplex PCR kit can also include a PCR kit 20 for determining the species and sex of Indonesian red arowanas.

In embodiments, the multiplex PCR kit can include a horizontal gel electrophoresis kit including 1.5% of agarose gel in 1X TBE buffer with 120 Voltage for separating, screening, and analysing the PCR amplified products. The multiplex PCR kit can include ethidium bromide in a concentration of 0.5 mg/ml in 1X TBE buffer. The multiplex PCR kit can also include a UV tray with a UV transilluminator to provide for observation of PCR amplified products under UV light.

In embodiments, the method of using the multiplex PCR kit for determining the species and sex of Asian red arowanas (i.e., Indonesian red arowanas) in one step or simultaneously can include mixing 3 µl of 25 ng/ml genomic DNA sample with 10.95 µl of the Species and Sex Master Mix, and 0.05 µl 5 U/µl IMMOLASE™ DNA Polymerase (Bioline, UK).

In embodiments, a test with a positive control and negative control can be conducted in parallel with the genomic DNA sample by using the same ratio of
premix as described above for each of the positive control and the negative control. In embodiments, the quantity of genomic DNA can be increased under cycling conditions that can include: initial denaturation at about 95 °C for about 10 minutes; about 32 cycles of denaturation at 95 °C for about 30 seconds, annealing at an optimal annealing temperature of about 61 °C for about 30 second and extension at about 72 °C for about 30 seconds; and a final extension at about 72 °C for about 7 minutes.

In embodiments, the PCR amplified product, produced from increasing the genomic DNA sample, can be separated using horizontal electrophoresis with 1.5% of agarose gel in 1 X TBE buffer with 120 Voltage for 25 minutes. Subsequently, the agarose gel can be strained using ethidium bromide in a concentration of 0.5 mg/ml in 1 X TBE buffer. The PCR amplified product can then be analysed in a UV-tray by observation under UV light provided by a UV transilluminator. If the 125 bp DNA band appears, the subject is an Asian red arowana (i.e., Indonesian red arowana). If the 320 bp DNA band appears, the subject is a female Asian red arowana (i.e., female Indonesian red arowana).

EXAMPLES

Example 1: Sex Determination of Indonesian Red Arowanas

Referring to FIG. 4, tissue samples were collected from the fresh gills of 12 male Indonesian red arowanas (M1 to M12) and 18 female Indonesian red arowanas (F1 to F19). DNA samples were isolated from the tissue samples (collected from the fresh gills) of all the male and female Indonesian red arowanas using the phenol-chloroform method. The isolated DNA samples derived from the fresh gills are also designated as "Gi" in FIG. 2. Additionally, for the female Indonesian red arowanas F13 to F18, a QIAamp DNA Stool Mini Kit (QIAGEN, Hilden, Germany) was used to isolate DNA samples from frozen stool samples. The isolated DNA samples derived from the frozen stool samples are designated as "Fe" in FIG. 2, while "Ma" in FIG. 4 is the 100 bp DNA ladder.
The sex of all the male and female Indonesian red arowanas was determined by visual inspection of the dissected gonad and by histological analysis of the dissected gonad.

Using the PCR primer pair of DSX66A and DSX66B, all 30 Indonesian red arowana individuals of known sex were screened. The PCR reaction mix included 0.5 µl of 10 µM DSX66A (PCR primer); 0.5 µl of 10 µM DSX66B (PCR primer); 1.25 µl of 10X PCR ImmoBuffer; 0.375 µl of 50 mM MgCl₂; 0.25 µl of 10 mM dNTP (deoxyribonucleotide triphosphate); 8.075 µl of ddH₂O (double-distilled water); 0.05 µl of 5 U/µl IMMOLASE™ DNA Polymerase (Bioline, UK); and 3 µl of 25 ng/µl isolated genomic DNA.

The PCR conditions included an initial denaturation at 95 °C for 10 minutes; 35 cycles of denaturation at 95 °C for 30 seconds, annealing at an optimal annealing temperature of 59 °C for 30 seconds and extension at 72 °C for 30 seconds; a final extension step at 72 °C for 10 minutes; and a hold step at 4 °C.

PCR was achieved using a temperature-gradient PCR machine (Veriti® Thermal Cycler, Applied Biosystems®, USA). The PCR products were separate and analysed using agarose gel electrophoresis. The PCR products were separated on 1.5% agarose gel.

PCR amplification results with the specific PCR reaction mix and specific PCR cycling conditions indicated a 320 bp DNA band that appeared in all 18 female Indonesian red arowanas and that was absent from all 12 male Indonesian red arowanas. Thus, the total accuracy rate of molecular sexing of Indonesian red arowanas is 100% using the specific PCR reaction mix and specific PCR cycling conditions designed by the inventors of the present disclosure. Furthermore, for the female Indonesian red arowanas F13 to F18, the genomic DNA isolated from tissue and the genomic DNA isolated from stool all generated the 320 bp DNA band, which
demonstrates that genomic DNA isolated from stool can be effectively used in the molecular sexing of Indonesian red arowanas and other arowanas.

The sensitivity test showed that a concentration of 15 nanogram/ml is a threshold concentration that can show the DNA bands clearly.

**Example 2: Species and Sex Determination of Indonesian Red Arowanas in One Step or Simultaneously using Multiplex PCR**

Referring to FIG. 5, tissue samples were collected from the fresh gills of 38 male Indonesian red arowanas (M1 to M38) and 26 female Indonesian red arowanas (F1 to F26). DNA samples were isolated from the tissue samples (collected from the fresh gills) of all the male and female-Indonesian red arowanas using the phenol-chloroform method. "Ma" in FIG. 3 is the 100 bp DNA ladder.

The sex of all the male and female Indonesian red arowanas was determined by visual inspection of the dissected gonad and by histological analysis of the dissected gonad.

Using the two PCR primer pairs of DSX66A/DSX66B and DSX06A/DSX06B, all 64 Indonesian red arowana individuals of known sex were screened. The multiplex PCR reaction mix for species and sex determination of Indonesian red arowanas in one step or simultaneously included: 0.5 µl of 10 µM DSX66A; 0.5 µl of 10 µM DSX66B; 0.25 µl of 10 µM DSX06A; 0.25 µl of 10 µM DSX06B; 1.25 µl of 10X ImmoBuffer; 0.375 µl of 50 mM MgCl2; 0.25 µl of 10 mM dNTP (deoxyribonucleotide triphosphate); 7.575 µl of double-distilled water; 0.05 µl of 5 U/µl IMMOLASE™ DNA Polymerase (Bioline, UK); and 3 µl of 25 ng/µl isolated genomic DNA.

The multiplex PCR cycling conditions for species and sex determination of Indonesian red arowanas in one step or simultaneously included: an initial
denaturation at 95 °C for 10 minutes; 32 cycles of denaturation at 95 °C for 30 seconds, annealing at an optimal annealing temperature of 61 °C for 30 seconds and extension at 72 °C for 30 seconds; a final extension step at 72 °C for 10 minutes; and a hold step at 4 °C.

PCR was achieved using a temperature-gradient PCR machine (Veriti® Thermal Cycler, Applied Biosystems®, USA). The PCR products were separate and analysed using agarose gel electrophoresis. The PCR products were separated on 1.5% agarose gel.

PCR amplification results with the specific multiplex PCR reaction mix and multiplex specific PCR cycling conditions indicated a JL25_bp_DNA band—that appeared in all 64 Indonesian red arowana individuals. Further, PCR amplification results with the specific multiplex PCR reaction mix and multiplex specific PCR cycling conditions indicated a 320 bp DNA band that appeared in all 26 female Indonesian red arowanas and that was absent from all 38 male Indonesian red arowanas. Thus, the total accuracy rate of multiplex PCR species and sex determination of Indonesian red arowanas is 100% for both species determination and sex determination using the specific multiplex PCR reaction mix and specific multiplex PCR cycling conditions designed by the inventors of the present disclosure.

Example 3: Sex Determination of Asian Arowanas

Referring to FIG. 6, tissue samples were collected from the fresh gills of 15 male Asian arowanas and 31 female Asian arowanas. The males are designated as "M" and females are designated as "F". M1-M12 and F1-F18 represent Indonesian red arowanas, M13-M15 and F19-F29 represent Malaysian gold arowanas, F30 represents an Asian green arowana, and F31 represents an Asian black arowana.

DNA samples were isolated from the tissue samples (collected from the fresh gills) of all the male and female arowanas using the phenol-chloroform method. The
isolated DNA samples derived from the fresh gills are also designated as "Gi" in FIG. 6. Additionally, for the female Indonesian red arowanas F13 to F18, a QIAamp DNA Stool Mini Kit (QIAGEN, Hilden, Germany) was used to isolate DNA samples from frozen stool samples, which are designated as "Fe" in FIG. 6. Further, for the male Malaysian gold arowana M15, a QIAamp DNA Stool Mini Kit (QIAGEN, Hilden, Germany) was used to isolate DNA samples from a frozen stool, frozen mucus, and from a gonad. The frozen stool DNA sample is designated as "Fe", the frozen mucus DNA sample is designated as "Mu", and the gonad DNA sample is designated as "Gon" in FIG. 6. Lastly, "Ma" is the 100 bp DNA ladder.

Using the PCR primer pair of Arol-F and DSX06B, all 46 arowana individuals of known sex were screened. The PCR reaction mix included: 0.5 µl of 10 µM Aro1-F (PCR primer); 0.5 µl of 10 µM DSX06B (PCR primer); 2.5 µl of 10X PCR Buffer with 15 mM MgCl₂; 2.00 µl of 1.25 mM dNTP (deoxyribonucleotide triphosphate); 17.20 µl of ddH₂O (double-distilled water); 0.30 µl of 5 U/µl RBC Taq DNA Polymerase; and about 2 µl of 25 ng/µl isolated genomic DNA.

The PCR cycling conditions included: an initial denaturation at 95 °C for 11 minutes; 35 cycles of denaturation at 94 °C for 1 minute, annealing at an optimal annealing temperature of 54 °C for 1 minute and extension at 72 °C for 1 minute; a final extension step at 72 °C for 10 minutes; and a hold step at 4 °C for an indefinite period of time.

PCR was achieved using a temperature-gradient PCR machine (Veriti ® Thermal Cycler, Applied Biosystems ®, USA). The PCR products were separate and analysed using agarose gel electrophoresis. The PCR products were separated on 1.5% agarose gel.

PCR amplification results with the specific PCR reaction mix and specific PCR cycling conditions indicated a 900 bp DNA band that appeared in all 31 female Asian
arowanas and that was absent from all 15 male Asian arowanas. Thus, the total accuracy rate of molecular sexing of Asian arowanas is 100% using the specific PCR reaction mix and specific PCR cycling conditions designed by the inventors of the present disclosure. Furthermore, for the female Indonesian red arowanas F13 to F18, the genomic DNA isolated from tissue and the genomic DNA isolated from stool all generated the 900 bp DNA band, which demonstrates that genomic DNA isolated from stool can be effectively used in the molecular sexing of Asian arowanas. Also, for the male Malaysian gold arowana M15, the genomic DNA isolated from tissue, stool, mucus, and sex organ did not generate the 900 bp DNA band.

While various aspects and embodiments have been disclosed herein, it will be apparent that various other modifications and adaptations of the invention will be apparent to the person skilled in the art after reading the foregoing disclosure without departing from the spirit and scope of the invention and it is intended that all such modifications and adaptations come within the scope of the appended claims. The various aspects and embodiments disclosed herein are for purposes of illustration and are not intended to be limiting, with the true scope and spirit of the invention being indicated by the appended claims.

REFERENCE

CLAIMS

1. A method of determining the sex of Indonesian red arowanas comprising:
   providing one or more Indonesian red arowanas;
   isolating one or more genomic DNA samples from the one or more Indonesian red
   arowanas; and
   identifying a sex-specific DNA marker for Indonesian red arowanas by using
   Polymerase Chain Reaction with a first primer and second primer.

2. The method of claim 1, wherein the identifying of the sex-specific DNA marker
   comprises amplifying the sex-specific DNA marker.

3. The method of claim 1 or 2, wherein the method has an accuracy of at least 83%
   for determining the sex of the one or more Indonesian red arowanas using the
   sex-specific DNA marker.

4. The method of any one of claims 1-3, wherein the method has an accuracy of at
   least 90% for determining the sex of the one or more Indonesian red arowanas using the
   sex-specific DNA marker.

5. The method of any of claims 1-4, wherein the method has an accuracy of at least
   95% for determining the sex of the one or more Indonesian red arowanas using the
   sex-specific DNA marker.

6. The method of any of claims 1-5, wherein the method has an accuracy of at least
   99% for determining the sex of the one or more Indonesian red arowanas using the
   sex-specific DNA marker.

7. The method of any of claims 1-6, wherein the method has an accuracy of about
   100% for determining the sex of the one or more Indonesian red arowanas using
   the sex-specific DNA marker.
8. The method of any one claims 1-7, wherein the sex-specific DNA marker is a female-specific DNA marker.

9. The method of claim 8, wherein the female-specific DNA marker comprises about 320 nucleotides.

10. The method of claim 8 or 9, wherein the female-specific DNA marker is at least 98% homologous with SEQ ID. 1.

11. The method of any one of claims 1-10, wherein the first primer comprises an oligonucleotide sequence of 5' TAACTCAAAGTAGAATAGAACAATG3'.

12. The method of any one of claims 1-11, wherein the second primer comprises an oligonucleotide sequence of 5' AATTCAAGGGAACTGACTCTA3'.

13. The method of any one of claims 1-12, wherein the one or more genomic DNA samples are isolated from a DNA source selected from the group consisting of muscle, gill, fin, mucus, stool and a combination of one or more thereof.

14. The method of any one of claims 1-13, wherein the one or more genomic DNA samples are isolated from stool.

15. The method of any one of claims 1-14, wherein the identifying of the sex-specific DNA marker further comprises the use of a PCR reaction mix and the use of PCR cycling conditions.

16. The method of claim 15, wherein the PCR reaction mix comprises: 0.5 µl of 10 µM of the first primer; 0.5 µl of 10 µM of the second primer; 1.25 µl of 10X PCR ImmoBuffer; 0.375 µl of 50 mM MgCl₂; 0.25 µl of 10 mM dNTP (deoxyribonucleotide triphosphate); 8.075 µl of double-distilled water; 0.05 µl of
5 U/µl IMMOLASE™ DNA Polymerase (Bioline, UK); and 3 µl of 25 ng/µl isolated genomic DNA.

17. The method of claim 15 or 16, wherein the PCR cycling conditions comprise: an initial denaturation at about 95 °C for about 10 minutes; about 35 cycles of denaturation at about 95 °C for about 30 seconds, annealing at an optimal annealing temperature of about 59 °C for about 30 seconds and extension at about 72 °C for about 30 seconds; a final extension step at about 72 °C for about 10 minutes; and a hold step at about 4 °C.

18. The method of any one of claims 1-14, further comprising:

- identifying a species-associated DNA marker by using Polymerase Chain Reaction with a third primer and fourth primer,

wherein the third primer comprises an oligonucleotide sequence of 5' ACACAGTGAGGTAACGATCT3',

wherein the fourth primer comprises an oligonucleotide sequence of 5' GAAACCAGGTGGACAGGTC3',

wherein the identifying of the species-associated DNA marker comprises amplifying the species-associated DNA marker, and

wherein the species-associated DNA marker is at least 98% homologous with SEQ ID. 2.

19. The method of claim 18, wherein the identifying of the sex-specific DNA marker and the identifying of the species-associated DNA marker further comprise the use of a PCR reaction mix and the use of PCR cycling conditions.

20. The method of claim 19, wherein the PCR reaction mix comprises: 0.5 µl of 10 µM of the first primer; 0.5 µl of 10 µM of the second primer; 0.25 µl of 10 µM of the third primer; 0.25 µl of 10 µM of the fourth primer; 1.25 µl of 10X PCR ImmoBuffer; 0.375 µl of 50 mM MgCl2; 0.25 µl of 10 mM dNTP
(deoxyribonucleotide triphosphate); 7.575 µl of double-distilled water; 0.05 µl of 5 U/µl IMMOLASE™ DNA Polymerase (Bioline, UK); and 3 µl of 25 ng/µl isolated genomic DNA.

21. The method of claim 19 or 20, wherein the PCR cycling conditions comprise: an initial denaturation at 95 °C for 10 minutes; 32 cycles of denaturation at 95 °C for 30 seconds, annealing at an optimal annealing temperature of 61 °C for 30 seconds and extension at 72 °C for 30 seconds; a final extension step at 72 °C for 10 minutes; and a hold step at 4 °C.

22. The method of any one of claims 18-21, further comprising: confirming that the one or more isolated genomic DNA samples are from Indonesian red arowanas rather than from contaminant DNA from microorganisms, contaminant DNA from animals used as fish feed or a combination thereof.

23. A kit for use in determining the sex of one or more Indonesian red arowanas comprising: a first primer comprising an oligonucleotide sequence of 5' TAACTCAAAGTGAATAGAACAATG'; a second primer comprising an oligonucleotide sequence of 5' AATTCAAGGAACTGATGACTCTA'; and IMMOLASE™ DNA Polymerase (Bioline, UK).

24. A kit for use in simultaneously determining the species and sex of one or more Indonesian red Arowanas comprising: a first primer comprising an oligonucleotide sequence of 5' TAACTCAAAGTGAATAGAACAATG'; a second primer comprising an oligonucleotide sequence of 5' AATTCAAGGAACTGATGACTCTA';
a third primer comprising an oligonucleotide sequence of 5' ACACAGTGCGAGGTAACAGAATCT3',
a fourth primer comprising an oligonucleotide sequence of 5' GAAACCAGAGTTGGGACAGGTC3', and
IMMOLASE™ DNA Polymerase (Bioline, UK).
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**FIG. 2**
A. CLASSIFICATION OF SUBJECT MATTER
C12Q 1/68(2006.01)i, C12N 15/11(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q 1/68; C12N 15/11

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & Keywords: Indonesian red arowanas, PCR, sex-specific DNA marker

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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<td>X</td>
<td>YUE et al., 'A strain specific and a sex associated STS marker for Asian arowana' (Sc leporipes formosus, Ost eogl oss idae)' Aquaculture Research, Vol.34, pp.951-957 (2003)</td>
<td>1-3, 23-24</td>
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<td>A</td>
<td>MANOHARAN et al., 'Isolation and characterisation of novel microsatellite markers in commercial selected golden Malaysian arowana fish, Sel eropages for mos (Ost eogl oss idae)' Genetics and Molecular Research, Vol.10, No.2, pp.712-716 (2011)</td>
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Further documents are listed in the continuation of Box C. See patent family annex.

* Special categories of cited documents:
  "A" document defining the general state of the art which is not considered to be of particular relevance
  "E" earlier application or patent but published on or after the international filing date
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  "O" document referring to an oral disclosure, use, exhibition or other means
  "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search
08 October 2013 (08.10.2013)

Date of mailing of the international search report
10 October 2013 (10.10.2013)

Name and mailing address of the ISA/KR
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Form PCT/ISA/210 (second sheet) (July 2009)
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<th>Citation</th>
<th>Relevant to claim No.</th>
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**Box No. II   Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [ ] Claims Nos.:
   - because they relate to subject matter not required to be searched by this Authority, namely:

2. [x] Claims Nos.: 9,16,19-20
   - because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
     - The claims 9,16 and 19-20 do not comply with PCT Article 6 because they are referring to the multiple dependent claims which do not comply with PCT Rule 6.4(a).

3. [x] Claims Nos.: 4-8,10-15,17-18,21-22
   - because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box No. III   Observations where unity of invention is lacking (Continuation of item 3 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. [x] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [x] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [x] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

- [ ] The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- [x] The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- [ ] No protest accompanied the payment of additional search fees.
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