Schlussbericht

Zahlungsempfänger:

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Vorhaben:

Development of an identification method for and a genetic assessment of Danube River (and Black Sea) sturgeon stocks as a prerequisite for sustainable fisheries and conservation management (DASTMAP)

Verbund: ERA-Net COFASP

Laufzeit: 01.04.2015 bis 31.10.2018

Berichtszeitraum: 01.04.2015 bis 31.10.2018

Berlin, den 31.01.2019
Ort, Datum

Dr. Klaus Kohlmann (Projektkoordinator)

Kurzfassung

Störe - die Lieferanten des teuer gehandelten Kaviars – sind lebende Fossilien, die die Erde seit etwa 250 Millionen Jahren bevölkern. Durch den Verlust von Lebensräumen und Überfischung gehören sie mittlerweile zu den weltweit am stärksten gefährdeten Fischarten. Ihr Rückgang ist besonders deutlich in der Donau und im Schwarzen Meer zu beobachten. Die dort heimischen Populationen des Sternhausens (*Acipenser stellatus)*, des Donaustörs (*A. gueldenstaedtii)*, des riesigen Hausen (*Huso huso)* und des Sterlets (*A. ruthenus)* stellen die letzten, sich selbst erhaltenden Störbestände in Europa dar. Aber auch sie sind akut gefährdet, obwohl oder auch weil sie noch bis vor 10 Jahren befischt wurden. Für effektive Schutzmaßnahmen dieser Wildbestände sind u.a. Kenntnisse ihrer genetischen Diversität und Strukuren erforderlich. Um diese Wissenslücken zu schließen, wurden im DASTMAP-Projekt mit Partnern aus Deutschland, Rumänien und der Türkei abgestimmte, genetische Analysemethoden entwickelt. Darauf aufbauende Untersuchungen zeigten, dass in der Unteren Donau vom Sterlet noch mindestens zwei und vom Hausen drei Subpopulationen vorkommen. Als Warnsignal ist zu werten, dass bei beiden Arten schon ein hoher Grad an Vermischung nachweisbar ist. Vergleichbare Ergebnisse werden auch für den Sternhausen erwartet. Besonders dramatisch ist die Situation bereits beim Donaustör, bei dem es wegen seiner extremen Seltenheit nicht gelang, eine ausreichende Anzahl an Proben zu beschaffen. Aus den gewonnenen genetischen Daten lassen sich Schlussfolgerungen für das Bestandsmanagement der vier Arten ableiten: Oberste Priorität hat die Wiederherstellung der Wandermöglichkeiten und der Funktionalität ehemaliger Laichplätze. Dadurch könnte die beobachtete Vermischung genetischer Linien innerhalb der Arten vermieden und die Erhaltung ihrer genetischen Integrität gewährleistet werden. Als zeitlich begrenzte Zwischenlösung können *ex situ* Laichfischbestände dienen, die jedoch repräsentativ für die Wildpopulationen sein müssen, um für Besatzzwecke geeignet zu sein. Solche Maßnahmen sind Teil des aktuellen Aktionsplans für die europäischen Störarten, für den das Projekt wesentliche Grundlagen erarbeitet hat. Die Umsetzung dieser Strategie ist unumgänglich, um das Aussterben der Arten in Europa zu verhindern.

Summary

Sturgeons – the producers of the high priced caviar – are living fossils inhabiting Earth for approx. 250 million years. Today, they are among the most endangered fish species worldwide due to habitat loss and overfishing. Their decline becomes highly evident in the Danube River and the Black Sea. In these regions the native populations of the stellate sturgeon (*Acipenser stellatus*), the Russian sturgeon (*A. gueldenstaedtii*), the giant beluga sturgeon (*Huso huso*), and the sterlet sturgeon (*A. ruthenus*) represent the last self-sustaining sturgeon stocks of Europe. Despite the fact (or because) they were heavily fished until 10 years ago they are critically endangered today. Effective conservation measures for the few remaining wild populations require, among other immediate measures, knowledge on their genetic diversity and structures. In order to close these knowledge gaps the DASTMAP project partners from Germany, Romania and Turkey developed harmonized methods of genetic analyses. Subsequent studies have shown that at least two sub-populations of sterlet sturgeon and three sub-populations of beluga sturgeon are still existing in the Lower Danube River. The observed high degree of admixture between the sub-populations of both species must be interpreted as a warning signal. Comparable results are also expected for stellate sturgeon. The status of Russian sturgeon is particularly dramatic because a suitable number of samples from this species could not be acquired due to its extreme scarcity. From the genetic data obtained the following conclusions are drawn for the management of the four sturgeon species: The restoration of migration routes and functionality of historic spawning grounds has highest priority resulting in avoidance of the observed intraspecific admixture of distinct genetic lineages and ensuring their genetic integrity. *Ex situ* broodstocks should be established as an interim solution. These must represent the genetic diversity of the wild populations in order to be suitable for (re)stocking. Such measures are part of the Pan-European action plan for sturgeon species, to which the project provided essential prerequisites. The implementation of this strategy is inevitable to prevent the extinction of these species in Europe.

1. Beitrag des Ergebnisses zu den förderpolitischen Zielen, auch zwecks Evaluierung von Förderprogrammen.

   Das Vorhaben DASTMAP wurde im Rahmen des ERA-Net COFASP gefördert. Eines der Hauptziele von COFASP war die Schaffung der Basis für eine nachhaltige Nutzung fischereilicher Ressourcen entsprechend des Vorsorgeprinzips. Dafür ist die Kenntnis der Bestände, die einer Nutzung und dem Management unterliegen, vor allem hinsichtlich ihrer genetischen Diversität und Strukturen, erforderlich. Von besonderer Bedeutung ist dieser Aspekt für Fischarten, die in ihrer Existenz bedroht sind, da hier etwaige Stützungsmaßnahmen auf die relevanten Managementeinheiten auszurichten sind. Zu diesen gefährdeten Arten zählen auch die Störe der Unteren Donau und des Schwarzen Meeres.
   Das internationale Konsortium bestehend aus deutschen, rumänischen und türkischen Partnern leistet mit den Ergebnissen des DASTMAP Vorhabens einen Beitrag zu diesen Zielen, indem:
   - genetische Marker (Mikrosatelliten-Loci und mitochondriale DNA-Sequenzen) für die vier Störarten *Acipenser stellatus*, *Acipenser ruthenus*, *Acipenser gueldenstaedtii* und *Huso huso* entwickelt wurden,
   - diese Marker für die Untersuchung der genetischen Diversität und der Populationsstrukturen der Störarten eingesetzt wurden, und
   - darauf aufbauend Empfehlungen für ein verbessertes Management und Artenschutzmaßnahmen abgeleitet wurden.

2. Aufzählung der wichtigsten wissenschaftlich-technischen Ergebnisse des Vorhabens im Vergleich zu den ursprünglichen Zielen, die erreichten Nebenergebnisse und die gesammelten wesentlichen Erfahrungen.

   Schwerpunkte der deutschen Arbeiten im Vorhaben DASTMAP waren:
   - die Isolierung von artspezifischen Mikrosatelliten-Loci aus Genombibliotheken der vier Störarten *Acipenser stellatus*, *Acipenser ruthenus*, *Acipenser gueldenstaedtii* und *Huso huso* durch einen Unterauftragnehmer (WP 2),
   - die Entwicklung von Multiplex-PCR Sets bestehend aus 12-15 Mikrosatelliten für jede der vier Störarten (WP 3),

- die Anwendung dieser Mikrosatelliten bei der Genotypisierung von Störproben aus der Unteren Donau in Zusammenarbeit mit dem rumänischen Projektpartner vom Danube Delta National Institute, DDNI (WP 4) und
- die Mitwirkung an der populationsgenetischen Analyse der gewonnenen Daten mit dem Ziel der Definition von Management-Einheiten für eine nachhaltige Fischerei und den Artenschutz (WP 8).

Aufzählung der wichtigsten wissenschaftlich-technischen Ergebnisse:

- WP 2:
  Vom Unterauftragnehmer (GenoScreen, Lille, Frankreich) wurden geliefert:

Störart	Anzahl der DNA-Sequenzen mit Mikrosatelliten-Loci	Anzahl validierter PCR-Primerpaare
Acipenser stellatus	6.416	253
Acipenser ruthenus	3.942	161
Acipenser gueldenstaedtii	5.752	198
Huso huso	2.898	121

  Damit wurde die ursprüngliche Zielstellung von 100 validierten PCR-Primerpaaren je Störart in jedem der vier Fälle erreicht und in drei von vier Fällen erheblich überschritten.

- WP 3:
  Aus den in WP 2 gelieferten, validierten PCR-Primerpaaren wurden je Störart 60-70 potentiell geeignete Paare ausgewählt und an Zuchtfischen aus einer deutschen Störfarm getestet. Im Ergebnis konnten die folgenden Multiplex-PCR Sets etabliert werden:

Störart	Anzahl der Multiplex-PCR Sets	Gesamtzahl der enthaltenen Loci
Acipenser stellatus	6	18
Acipenser ruthenus	5	15
Acipenser gueldenstaedtii	4	12
Huso huso	5	12

  Damit wurde die ursprüngliche Zielstellung der Entwicklung von Multiplex-PCR Sets bestehend aus 12-15 Mikrosatelliten-Loci je Störart erreicht bzw. für *A.*

stellatus überboten. Die DNA-Sequenzen aller *de novo* isolierten Loci wurden in der NCBI GenBank deponiert und sind damit frei zugänglich.
Wegen mangelnder Variabilität der getesteten artspezifischen Loci musste bei *A. ruthenus* und *H. huso* jedoch zusätzlich auf Mikrosatelliten zurückgegriffen werden, die von verwandten Störarten stammen (s. auch Punkt 4). Für jede der vier Störarten wurde ein entsprechender Methoden-Artikel veröffentlicht (s. Punkt 7 und Kopien im Anhang).

- WP 4:
  Unter Verwendung der in WP 3 entwickelten Multiplex-PCR Sets konnte die folgende Anzahl rumänischer Störproben genotypisiert werden:
  Acipenser gueldenstaedtii: 11 Adulte aus Wildfängen und 20 Juvenile aus einer Fischzuchtanstalt.
  Acipenser ruthenus: 151 Individuen.
  Acipenser stellatus: werden vom DDNI genotypisiert (s. unten).
  Huso huso: 183 Individuen.

  Die geringe verfügbare Probenzahl von *Acipenser gueldenstaedtii* resultiert aus der inzwischen extremen Seltenheit dieser Störart in der Unteren Donau. In den vergangenen Jahren wurden im Monitoring nur wenige Einzeltiere erfasst, auch durch die Kontakte in die Fischerei konnten keine zusätzlichen Proben für das Vorhaben beschafft werden. Die in WP 8 geplante Untersuchung der Populationsstruktur der Art war damit nicht möglich. Alternativ wurde deshalb ein genetischer Vergleich dieser Proben mit Farmstören aus Deutschland, Rumänien und der Türkei durchgeführt (s. Ergebnisse WP 8). Da durch das Vorhaben die genetischen Untersuchungsmethoden etabliert sind (s. WP 3), ist der rumänische Projektpartner in die Lage versetzt worden, die bisher gewonnenen Datensätze nach Projektende durch weitere Proben zu ergänzen und das ursprüngliche Projektziel doch noch zu erreichen.

  Wegen zeitlicher Verzögerungen bei der Entwicklung der Multiplex-PCR Sets in WP 3 (s. Erläuterungen unter Punkt 3) und um weitere Verzögerungen zu vermeiden, einigten sich die deutschen und rumänischen Partner darauf, dass sämtliche Proben von *Acipenser stellatus* am DDNI genotypisiert werden sollen anstelle der geplanten Typisierung am IGB Berlin.

- WP 8:

  Die populationsgenetischen Untersuchungen des Sterlets *(Acipenser ruthenus)*, die im Rahmen des Vorhabens realisiert wurden, zeigten für die Wildpopulation aus dem rumänischen Abschnitt der Donau genetisch signifikante Unterschiede zu einem Zuchtbestand („blau“ in Abb. 1) aus einer deutschen Störfarm, der als Vergleichsgruppe herangezogen wurde. Innerhalb der rumänischen Sterlet-Population konnten mit Hilfe der neu etablierten Mikrosatelliten-Loci drei genetisch unterschiedliche Cluster identifiziert werden. Zwei dieser Cluster („rot“ und „grün“ in Abb. 1) dominierten, wiesen neben genetisch „reinen“ Individuen aber auch eine hohe Anzahl an Hybridtieren zwischen diesen beiden Clustern auf. Der dritte Cluster („gelb“ in Abb. 1) bestand aus nur zwei Individuen, die unter Hinzuziehung mitochondrialer DNA-Sequenzen (Cytochrom b und D-Loop) als Arthybriden aus weiblichen *A. stellatus* und männlichen *A. ruthenus* identifiziert werden konnten.

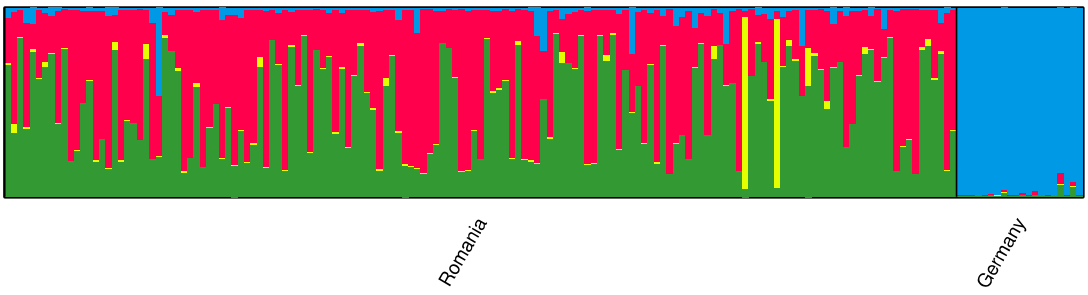

Abb. 1: Ergebnisse der mikrosatelliten-basierten Clusteranalyse des Sterlets, *Acipenser ruthenus*

Für die genetische Durchmischung ursprünglich getrennter Linien innerhalb der Art Sterlet (= intraspezifische Hybridisierung) kommen mehrere Ursachen infrage. So ist eine stromabgerichtete Wanderung der Tiere während ihrer Jugend (vor Eintritt in die Geschlechtsreife) typisch. Diese Wanderung führt aktuell zu einer Verdriftung über die Stauhaltungen des Eisernen Tores, einem Komplex aus Wasserkraftanlagen, die stromab bedingt passierbar sind, aber eine unüberwindbare Hürde für den Aufstieg darstellen. Somit findet eine Introgression von früher getrennten Beständen statt, die die beobachtete Durchmischung bedingen kann. Eine weitere Ursache kann die Einfuhr von gebietsfremden und damit genetisch verschiedenen Populationen der Art *A. ruthenus* sein. Zur Klärung dieser Frage sind jedoch weiterführende Untersuchungen erforderlich. Schließlich trägt zum Auftreten natürlicher intra- wie auch interspezifischer Hybriden auch der Verlust und daraus resultierende Mangel an Laichplätzen bei; die verbliebenen Laichplätze werden nun von

genetisch differenzierten Linien einer Art und gleichzeitig auch von verschiedenen Arten genutzt, was zu den beschriebenen Konsequenzen führt. Beide Prozesse (Hybridisierung innerhalb und zwischen Störarten) stellen ein ernstes Risiko für die Erhaltung der Wildpopulation des Sterlets dar.
Diese Ergebnisse wurden auf dem 26. Symposium „Deltas and Wetlands“ vom 16.-20. Mai 2018 in Tulcea, Rumänien als Vortrag präsentiert (s. Punkt 7).

Die auf den Mikrosatelliten-Loci basierenden populationsgenetischen Untersuchungen des Hausens, *Huso huso*, konnten die Individuen aus der rumänischen Unteren Donau drei genetischen Clustern zuordnen („orange“, „gelb“ und „blau“ in Abb. 2). Diese wiesen eine ungleichmäßige Verteilung zwischen den Jahren der Probennahme und einen hohen Grad der Durchmischung/Hybridisierung auf. Individuen des „orangen“ und „gelben“ Clusters dominierten z.B. im Jahr 2006, während Individuen des „blauen“ Clusters im Jahr 2010 vorherrschten. Die Individuen aus einer deutschen Störfarm gehörten alle zum „orangen“ Cluster, der in der Unteren Donau insgesamt seltener vorkam. Die wenigen aus türkischen Küstengewässern verfügbaren Proben (n=7) gehörten zum „blauen“ (n=4) oder „orangen“ (n=1) Cluster bzw. waren „admixed“ (n=2).

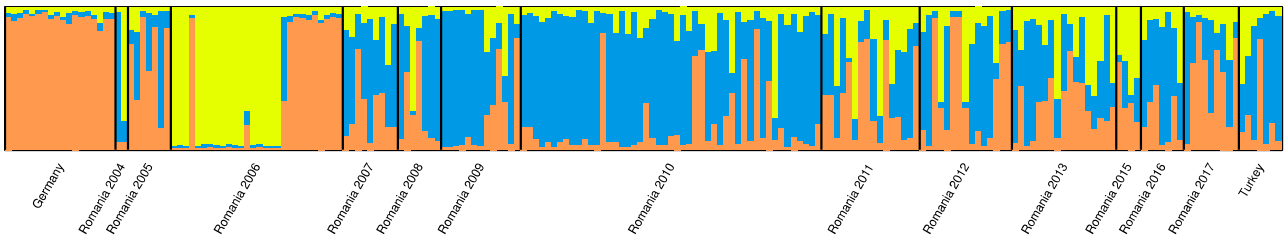

Abb. 2: Ergebnisse der mikrosatelliten-basierten Clusteranalyse des Hausens, *Huso huso*

Die Arbeitshypothese der Differenzierung des Bestandes des *H. huso* in vier genetisch identifizierbare Untergruppen in Anlehnung an Berg (1936), konnte durch die genetischen Untersuchungen nicht bestätigt werden. Ob dies darauf zurückzuführen ist, dass über die letzten 50 Jahre bereits eine intraspezifische Hybridisierung voranschreitet, ist aktuell noch unklar. Einkreuzungen aus streunenden Beständen, die durch massive Besatzmaßnahmen gestützt wurden, sind ebenfalls möglich. Dies ist vor allem bedenklich, da auch bei *H. huso* Tiere aus dem Kaspi-Einzugsgebiet für den Besatz genutzt wurden.

Die Kombination der Mikrosatelliten- mit den mtDNA-Daten wird momentan durch die rumänischen Projektpartner vorgenommen, die eine Verlängerung der Projektlaufzeit bis Ende Februar 2019 bei ihrem nationalen Projektträger beantragt und bewilligt bekommen haben; Ergebnisse liegen daher noch nicht vor.

Wie bereits erwähnt, konnten die populationsgenetischen Analysen basierend auf den entwickelten Multiplex-PCR Sets an *Acipenser gueldenstaedtii* aufgrund der unzureichenden Probenzahl nicht erfolgreich durchgeführt werden. Dennoch konnte beim Vergleich der wenigen Wildfänge mit Farm-Individuen gezeigt werden, dass die rumänischen Wild- und Farm-Individuen zu einer Gruppe gehören, die sich genetisch deutlich von einer zweiten Gruppe, bestehend aus deutschen und türkischen Farm-Individuen, unterscheidet (Abb. 3). Damit konnte die prinzipielle Eignung der entwickelten Multiplex-PCR Sets für die Beantwortung populationsgenetischer Fragestellungen nachgewiesen werden.

Abb. 3: Neighbor-Joining Dendrogramm der vier untersuchten *Acipenser gueldenstaedtii* Gruppen basierend auf der genetischen Distanz

Die populationsgenetischen Analysen an *Acipenser stellatus* unter Federführung der rumänischen Projektpartner liefen zum Zeitpunkt der Berichtserstellung noch, so dass Ergebnisse noch nicht verfügbar waren.

Eine wesentliche Schlußfolgerung aus den bereits vorliegenden und noch zu erwartenden populationsgenetischen Daten aller vier Störarten ist die dringende Notwendigkeit der Wiederherstellung der Wandermöglichkeiten und die Verifizierug der Intaktheit der historischen Laichplätze. Nur durch den ungehinderten Zugang zu diesen Laichplätzen – nicht nur in der Donau, sondern auch in türkischen Flüssen – würden die beobachteten Vermischungen/Hybridisierungen genetisch differenzierter Linien innerhalb

der Art, aber auch Hybridisierungen zwischen den Arten, vermieden und die Erhaltung der genetischen Integrität gewährleistet.
Als zeitlich begrenzte Zwischenlösung können *ex situ* Laichfischbestände dienen, deren Etablierung und Reproduktion jedoch die genetisch nachgewiesenen Subpopulationen berücksichtigen muss.

3. Darstellung und Erläuterung der Angemessenheit von Aufwand und Zeit

Die zusätzlich notwendig gewordenen Tests von artfremden Mikrosatelliten-Loci für die Genotypisierung von *Acipenser ruthenus* und *Huso huso* waren in der ursprünglichen Projektplanung nicht vorhersehbar. Sie führten zu einem erhöhten Aufwand an Laborarbeiten und Zeit. Um diesen zu kompensieren, einigten sich die deutschen und rumänischen Partner darauf, dass sämtliche Proben von *Acipenser stellatus* am DDNI anstelle des IGB Berlin genotypisiert werden sollten.
Insgesamt kann dadurch der Aufwand und die Zeit für die Erreichung der Projektziele des deutschen Partners als angemessen betrachtet werden. Vor dem Hintergrund der akuten Bedrohung des Erhaltes der drei wandernden Störarten im Schwarzen Meer und insbesondere in der Donau, als letztem Europäischen Gewässer mit selbst erhaltenden Populationen dieser Arten und den derzeit geplanten und anlaufenden Arterhaltungsmaßnahmen ist die Arbeit im Rahmen des Vorhabens sowohl zeitlich als auch inhaltlich zur rechten Zeit gekommen, um Vorsichtsmaßnahmen beim Aufbau der Elterntierbestände zu ergreifen. Weitere Klärungen zu Hintergründen der Ergebnisse, wie der Abgleich der genetischen Charakteristika zwischen Schwarzem und Kaspischen Meer sind dringend geboten, um zielführende Managementmaßnahmen vorbereiten zu können. Die Einbeziehung der lokalen Partner hat sich für das Gesamtvorhaben als sehr sinnvoll herausgestellt, da nur so eine Methodensicherheit und eine Akzeptanz der Ergebnisse sicherzustellen ist. Vor diesem Hintergrund waren die Projektmittel gut angelegtes Geld. Nachfolgende Vorhaben zur weiteren detaillierten Aufbereitung der Daten und für zusätzliche Unteruchungen sind bereits in internationaler Kooperation angelaufen.

4. Aufführen von Arbeiten, die zu keiner Lösung geführt haben.

   Wie bereits unter Punkt 2, Ergebnisse des WP 3 ausgeführt, waren die Tests von 60-70 artspezifischen PCR-Primerpaaren bei *Acipenser ruthenus* und *Huso huso* nur zum Teil erfolgreich: bei *Acipenser ruthenus* konnten nur sechs geeignete artspezifische Mikrosatelliten-Loci gefunden werden, bei *Huso huso* war es sogar nur ein Locus. Um auf die angestrebte Anzahl von mindestens 12 Mikrosatelliten je Störart zu kommen, mussten daher auch artfremde, von verwandten Störarten stammende Loci auf ihre Eignung getestet werden. Im Endergebnis konnte dadurch die Zielstellung bei beiden Arten dann doch erreicht und die in WP 8 geplanten populationsgenetischen Untersuchungen erfolgreich durchgeführt werden (s. Punkt 2)

5. Darstellung und Erläuterung der wissenschaftlichen und ggf. Anschlussfähigkeit für eine mögliche nächste Phase.

   Die vom deutschen Projektpartner für alle vier Störarten entwickelten Multiplex-PCR Sets wurden in internationalen, peer-reviewten Fachjournalen veröffentlicht (s. Punkt 7) und stehen damit für interessierte Anwender zur freien Verfügung. Neben der Untersuchung der Diversität und Struktur der noch existierenden Wildpopulationen der vier Störarten – wie im vorliegenden Projekt – könnten diese Mikrosatelliten-Loci auch bei Farmbeständen zur Anwendung kommen, z.B. für:

   - die Identifizierung genetisch geeigneter Laichfischbestände für Besatz- oder Wiedereinbürgerungsmaßnahmen,
   - die Etablierung von Zuchtprogrammen basierend auf den individuellen genetischen Profilen der Laichfische, oder
   - das Monitoring von genetischen Veränderungen im Zuchtbestand (u.a. Verlust von genetischer Variabilität durch genetische Drift und/oder Inzucht, Domestikationseffekte).

   Die deutschen und rumänischen DASTMAP-Projektpartner haben mit Beteiligung französischer Partner erfolgreich ein ERA-Net COFASP Nachfolgeprojekt [Titel: Genome-based approaches for improvement of aquaculture in two marine sturgeon species: Atlantic sturgeon (*Acipenser oxyrinchus*) and Beluga (*Huso huso*)] zur Erweiterung des Methodenspektrums und der Anwendung u.a. in der kommerziellen Aufzucht eingeworben. Von Seiten der DASTMAP-Projektpartner ist zudem geplant, gemeinsam mit italienischen Partnern eine Evaluierung verschiedener Herkünfte von *H.*

huso als Grundlage für die Identifikation genetisch geeigneter Herkünfte für ein Wiedereinbürgerungsvorhaben, durchzuführen.

6. War der Einsatz der Bundesmittel für die Erreichung des geplanten Vorhabenziels ursächlich oder wäre dieses Ziel auch ohne Bundesmittel erreicht worden (einschließlich Bewertung evtl. Mitnahmeeffekte)?

   Der Einsatz der Bundesmittel war für die Erreichung des geplanten Vorhabenziels essentiell. Abgesehen von den Personalkosten und der gerätetechnischen Laborausstattung standen keine weiteren institutseigenen Ressourcen oder Drittmittel für die Durchführung des Vorhabens zur Verfügung.

7. Präsentationsmöglichkeiten für mögliche Nutzer – z.B. Anwenderkonferenzen (soweit die Art des Vorhabens dies zulässt) und Darstellung der erfolgten und geplanten Veröffentlichungen des Ergebnisses.

   Die Projektergebnisse wurden bisher in Form von 5 Präsentationen auf internationalen Konferenzen und 4 Veröffentlichungen in internationalen, peer-reviewten Fachjournalen für mögliche Nutzer zur Verfügung gestellt.

   Konferenzbeiträge als Vortrag (V) bzw. Poster (P):

   - Devrim Memiş, Deniz Devrim Tosun, Güneş Yamaner, Gökhan Tunçelli, Jörn Gessner, (V): Recent status of sturgeon in the Sakarya River in Turkey. 8th International Symposium on Sturgeon. Wien, Österreich, 10.-16. September 2017.
   - Radu Suciu, Marian Paraschiv, Mitică Ciorpac, Dalia Onără, Elena Taflan, Daniela Holostenco, Stefan Hont, Marian Iani, Katarina Tosic, Jörn Geßner, Klaus Kohlmann, (P): A molecular and ecological approach to Lower Danube Beluga Sturgeon offspring during the last two decades. 8th International Symposium on Sturgeon. Wien, Österreich, 10.-16. September 2017.
   - Klaus Kohlmann, Mitică Ciorpac, Petra Kersten, Jörn Geßner, Elena Taflan, Katarina Tosic, Daniela Holostenco, Radu Suciu, (V): Genetic assessment of sterlet (*Acipenser ruthenus*) from a Lower Danube wild population using nuclear

and mitochondrial DNA markers. 26th Symposium ”Deltas and Wetlands”. Tulcea, Rumänien, 16.-20. Mai 2018.

- Klaus Kohlmann, Petra Kersten, Jörn Geßner, Oguzhan Eroglu, Sirin Firidin, Mitică Ciorpac, Elena Taflan, Radu Suciu, (P): Validation of 12 species-specific, tetrasomic microsatellite loci from the Russian sturgeon, *Acipenser gueldenstaedtii*, for genetic broodstock management. 26th Symposium ”Deltas and Wetlands”. Tulcea, Rumänien, 16.-20. Mai 2018.
- Jörn Geßner, Petra Kersten, Mitică Ciorpac, Elena Taflan, Klaus Kohlmann, (V): Prerequisites and practical implications for *ex situ* measures in the Danube Region. European Sturgeon Conference. Wien, Österreich, 8.-10. Juli 2018.

Erfolgte Veröffentlichungen:

- Klaus Kohlmann, Petra Kersten, Jörn Geßner, Dalia Onără, Elena Taflan, Radu Suciu (2017). Isolation and characterization of 18 polymorphic microsatellite markers for the critically endangered stellate sturgeon, *Acipenser stellatus*. European Journal of Wildlife Research 63: 75.
- Klaus Kohlmann, Petra Kersten, Jörn Geßner, Dalia Onără, Elena Taflan, Radu Suciu (2017). New microsatellite multiplex PCR sets for genetic studies of the sterlet sturgeon, *Acipenser ruthenus*. Environmental Biotechnology 13: 11-17.
- Klaus Kohlmann, Petra Kersten, Jörn Geßner, Oguzhan Eroglu, Sirin Firidin, Mitică Ciorpac, Elena Taflan, Radu Suciu (2018). Validation of 12 species-specific, tetrasomic microsatellite loci from the Russian sturgeon, *Acipenser gueldenstaedtii*, for genetic broodstock management. Aquaculture International 26: 1365-1376.
- Klaus Kohlmann, Mitică Ciorpac, Petra Kersten, Radu Suciu, Elena Taflan, Katarina Tosic, Daniela Holostenco, Jörn Geßner (2018). Development and assessment of a multiplex PCR assay for genetic analyses of microsatellite loci in beluga sturgeon, *Huso huso*. Genetics of Aquatic Organisms 2: 35-41.

Darüber hinaus sind weitere 3 peer-reviewte Artikel in internationalen Fachjournalen geplant, die sich mit der genetischen Diversität und den Populationsstrukturen der drei Störarten *A. ruthenus*, *A. stellatus* und *H. huso* in der Unteren Donau befassen. Ein vierter peer-reviewte Artikel soll zusammenfassend die Schlussfolgerungen und Empfehlungen des DASTMAP- Projektes für Schutzmaßnahmen und ein nachhaltiges Management der Stör-Populationen in Rumänien und der Türkei zum Inhalt haben.

Weitere Präsentationen auf wissenschaftlichen Konferenzen sind ebenfalls geplant, ihre Anzahl sowie Zeit und Ort geeigneter Konferenzen kann aber momentan noch nicht genauer benannt werden.

Anhang

Kopien der bisher aus dem DASTMAP Vorhaben hervorgegangenen Publikationen

Eur J Wildl Res (2017) 63: 75
DOI 10.1007/s10344-017-1132-3

METHODS PAPER

Isolation and characterization of 18 polymorphic microsatellite markers for the critically endangered stellate sturgeon, *Acipenser stellatus*

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Received: 27 February 2017 / Revised: 1 August 2017 / Accepted: 17 August 2017 / Published online: 25 August 2017

Abstract The stellate sturgeon, *Acipenser stellatus*, is a critically endangered fish species. Knowledge on its genetic diversity and population structure is urgently needed to enable the identification of management units in order to prevent extinction. Therefore, 18 species-specific, polymorphic microsatellite loci have been isolated using GS-FLX Titanium pyrosequencing, arranged into 6 multiplex PCR sets, and characterized in 52 individuals (20 farmed and 32 wild). The total number of alleles per locus varied between 3 and 36 with an average of 8.44. The wild individuals were more diverse with an average number of 8.17 alleles per locus than the farmed ones with 3.28 alleles per locus. Observed heterozygosities ranged from 0.050 to 0.950 in the farmed and from 0.094 to 0.969 in the wild individuals. Significant deviations from Hardy-Weinberg equilibrium were found at 3 loci of the farmed and 5 loci of the wild individuals. The two sturgeon groups were significantly differentiated (F_{ST} = 0.118). The high sensitivity and discriminatory power of the 18 loci were proven by a very low overall probability of identity for siblings (PIsib = 8.73×10^{-6}) and a high accuracy of self-classification (98%). Thus, these newly developed markers represent a valuable genetic toolbox to identify management units for species conservation and sustainable fisheries.

Keywords Conservation genetics · Fisheries management · Microsatellite · Multiplex PCR · Stellate sturgeon

The stellate sturgeon, *Acipenser stellatus* Pallas 1771, is an anadromous species of the genus Acipenseridae endemic to the Ponto-Caspian region. It is highly valued for its roe which is marketed as Sevruga caviar. Intensive legal and illegal fisheries, dramatic habitat loss due to dam construction, and navigation facilitation as well as decreased water quality contributed to the drastic decline of its populations. The IUCN (International Union for Conservation of Nature) Red List of Threatened Species classified the stellate sturgeon as "endangered" in 1996 but increased the threat level to "critically endangered" in 2010 (Qiwei 2010).

As has been described in several sturgeon species *A. stellatus* populations comprise up to four types (historically called "races") which differ in behavior (timing and distance of spawning migrations) and do not or only little overlap reproductively (Berg 1935). As a result of reproductive isolation and local adaptation to different spawning grounds, the four types may also differ genetically. However, this hypothesis has not been verified yet. Moreover, it is highly probable that some of these types are affected by anthropogenic alterations to a higher degree than others: long distance migrants, for example, face a higher probability of encountering migration barriers, thus reducing their reproductive success and increasing the risk of intraspecific hybridization. Therefore, such types or "races" should be prime targets for conservation measures. Attempts to preserve their historic genetic variability and differentiation require knowledge of the genetic substructure of the species based on suitable genetic markers.

Microsatellite loci are a popular marker type in a wide variety of genetic investigations in fishes (Liu and Cordes 2004) and already demonstrated their usefulness in other

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10344-017-1132-3) contains supplementary material, which is available to authorized users.

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sturgeon species, e.g., to study the evolution of ploidy and functional diploidization (Rajkov et al. 2014), to identify species and hybrids (Barmintseva and Mugue 2013), and to estimate genetic diversities, the degree of spatial population structuring (DeHaan et al. 2006), or effective population sizes (O'Leary et al. 2014). The aim of the present study was to isolate and characterize polymorphic loci in stellate sturgeon, which is considered as functionally diploid (Ludwig et al. 2001), to provide a genetic toolbox for conservation measures and sustainable fisheries. To the best of our knowledge, these are the first 18 microsatellites described from this species.

Fin clips were collected from 20 juvenile stellate sturgeons at a German fish farm to isolate total genomic DNA using the DNeasy Blood & Tissue Kit (QIAGEN) according to manufacturer's protocols. A pool of ten DNA isolates was sent to GenoScreen, Lille, France (www.genoscreen.fr), where 1 μg of the pooled DNA was used for the development of microsatellite libraries through 454 GS-FLX Titanium pyrosequencing as described in Malausa et al. (2011). The bioinformatics program QDD (Meglécz et al. 2010) was used to analyze sequences. QDD treats all steps from raw sequences until obtaining PCR primers: removing adapters and vectors, detection of microsatellites, redundancy and possible mobile element association, selection of sequences with target microsatellites and primer design by using BLAST (ftp://ftp.ncbi.nih.gov/blast/executables), Clustal W (Larkin et al. 2007), and Primer3 (Rozen and Skaletsky 2000) programs. Among 6416 sequences containing a microsatellite motif, 253 bioinformatically validated primer pairs were designed.

All sequences with validated primer pairs were ranked according to motif type (penta- > tetra- > tri- > di-nucleotide repeats), number of repeats (the higher the better), and PCR product size (> 100 bp) considering only sequences with perfect repeats. From this list, the 60 top ranking primer pairs were selected for the identification of suitable microsatellites (consistent amplification, ease to score, sufficient variability). Amplification protocols were developed for future use of PCR multiplex kits (QIAGEN) and a peqSTAR 96X Universal Gradient thermocycler (Peqlab). PCR primers were multiplexed (grouped) by the software MultiPLX, version 2.1 (Kaplinski et al. 2005). Three different dye labels (Atto 680, Cyanine 5, and DY-751) were assigned to forward primers. Genotyping of microsatellite loci was performed on an eight-capillary sequencer CEQ 8000 (Beckman Coulter) using the Fragment Analysis module of the GenomeLab™ GeXP Genetic Analysis System, version 10.2 (Beckman Coulter).

The microsatellite variability was initially examined in 20 farmed individuals but later on extended to 32 wild stellate sturgeons originating from the Romanian part of River Danube. All microsatellite genotypes were examined with the Micro-Checker software, version 2.2.3 (van Oosterhout et al. 2004), for scoring errors due to null alleles, stutter bands, and/or large allele dropout. Then, general parameters of microsatellite loci variability (number of alleles, number of private alleles, observed and expected heterozygosity) were calculated, and tests for significance of deviations from Hardy-Weinberg equilibrium and linkage disequilibrium were performed using GENEPOP 4.0 (Rousset 2008). The discriminatory power of loci (probability of identity = PI) was estimated with GENECAP version 1.4 (Wilberg and Dreher 2004) applying the more conservative measure of PI for siblings PIsib (Waits et al. 2001). Genetic differentiation between farmed and wild stellate sturgeons was tested by calculating the F_{ST}-value using FSTAT, version 2.9.3.2 (Goudet 2002). Additionally, the sensitivity of the microsatellite loci was examined by self-classification using the Bayesian method and "leave one out" procedure of the GeneClass software (Cornuet et al. 1999).

Out of the 60 PCR primer pairs, 54 (90%) amplified successfully using the following protocols: each PCR reaction mix consisted of 5.0 μl of master mix, 1.5 μl Q-solution, 1.0 μl DNA isolate, primers with concentrations as stated in Table 1, and PCR-grade water up to a final volume of 10.0 μl. The PCR program based on QIAGEN recommendations included an initial denaturation at 95 °C for 15 min followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C (multiplex sets 1 and 2) or 60 °C (multiplex sets 3 to 6) for 90 s, and extension at 72 °C for 60 s. A final extension at 60 °C lasted for 30 min. Among the 54 successfully amplified loci, 36 were either monomorphic, expressed only 2 alleles or displayed more than 2 peaks, and, therefore, were excluded from further analyses (basic information on the 42 discarded loci is provided in a supplementary Table S1).

The remaining 18 suitable loci could be arranged into 6 multiplex PCR sets (Table 1). Tests with the Micro-Checker software did not reveal any evidence for scoring errors due to stutter bands or large allele dropout. However, analyses indicated homozygote excess at locus *Ast57* in the farmed and at loci *Ast22* and *Ast25* in the wild stellate sturgeon meaning that null alleles might be present at these three loci. The total observed number of alleles per locus ranged between 3 and 36 with an average of 8.44. The wild individuals were more diverse with an average number of 8.17 alleles per locus than the farmed ones with 3.28 alleles per locus. Consequently, private alleles were almost exclusively found in the wild stellate sturgeons (Table 2). Significant linkage disequilibrium across all stellate sturgeons was detected for three pairs of loci: *Ast6* and *Ast30* ($P = 0.037$), *Ast25* and *Ast44* ($P = 0.038$), and *Ast48* and *Ast57* ($P = 0.029$). Observed heterozygosities ranged from 0.050 at loci *Ast14*, *Ast53*, and *Ast57* to 0.950 at locus *Ast25* in the farmed and from 0.094 at locus *Ast14* to 0.969 at locus *Ast30* in the wild individuals (Table 2). Significant deviations from Hardy-Weinberg equilibrium were found at three loci in farmed and five loci in wild stellate sturgeon (Table 2). Three out of these eight deviations were due to the possible presence

Table 1 Characteristics of 18 polymorphic microsatellite loci isolated from *Acipenser stellatus* and tested in 52 individuals

PCR multiplex set (annealing temperature)	Locus name	Forward primer dye label	Primer concentration (μM)	Primer sequence (5′-3′)	Repeat motif	Total number of alleles	Allele size range (bp)	Probability of identity for siblings (PIsib)
1 (57 °C)	*Ast26*	Atto 680	0.210	F: GCCATTGACGATTTCACAGA R: TGCTGCGCATTACATTAACC	AC	11	121–145	0.3898
	Ast22	DY-751	0.260	F: TTTTTGAATTGTCAAAGGCG R: CCAGTGTGCTTGTGAATGCT	TTC	15	193–235	0.3182
	Ast44	Cyanine 5	0.052	F: GGCTGTTTGTTGGAATGCTT R: AACCACGGGTGAACACATTT	GA	3	181–185	0.5794
2 (57 °C)	*Ast3*	Atto 680	0.170	F: TCTGAAGAAAGTGGAAGCCAA R: ACTATGGAATGTTCCAGCTGAT	AGAC	8	106–138	0.4904
	Ast57	DY-751	0.250	F: CCGTCCACAACTTGCATAAT R: GTTGCCACCAAGAAATCACA	AG	3	136–140	0.6089
	Ast53	Cyanine 5	0.040	F: TCCAATGGGTTTAGCTCAGG R: GTCATGCGTGAAGGATGCTA	CT	3	169–175	0.8944
3 (60 °C)	*Ast20*	Atto 680	0.140	F: GTGCTGGATCAAGAAATCGG R: GGCCTCCTCCTCTTCCTCT	CTT	6	127–145	0.7427
	Ast43	DY-751	0.230	F: CTCTCAGGCAGCTTACAGGC R: TTAGCAGCGAGCATTCCTTT	CT	5	156–172	0.4651
	Ast36	Cyanine 5	0.039	F: CAACAGCAAAGCAGGTTCTG R: GAGTGAGGAGTGCGCAACAT	AG	3	236–240	0.6419
4 (60 °C)	*Ast6*	Atto 680	0.180	F: AGCATTCTTTCTGTTTTACTGTGA R: AGATTGACTGCAATCCAGGG	CTTT	8	128–156	0.3891
	Ast14	DY-751	0.310	F: TGGTAAGGCTGGTCTGGTTC R: AACAAGGGTTCAAAACGTCG	TCC	3	151–157	0.9273
	Ast39	Cyanine 5	0.044	F: CGGAGGAGTGGGAAGAGAAT R: CCCAATGAAATCCTTCCAGA	AG	3	173–177	0.7178
5 (60 °C)	*Ast25*	Atto 680	0.230	F: CCCCAATTGTTCTTGGATGT R: TGTGCTACAGAGCACTTGATTCT	TG	19	145–195	0.3045
	Ast48	DY-751	0.290	F: TCTAAAATTGGAAGCTACAAAGC R: CAGAGGGAGCGTCTCAAAAG	TG	3	189–195	0.6543
	Ast30	Cyanine 5	0.045	F: GGTGTCCGTTGAGGGAGTAA R: AGGGCGGTTAGTCTCATCTG	CT	36	204–338	0.3012
6 (60 °C)	*Ast51*	Atto 680	0.105	F: CCGTTCCAGCTAATGGATCA R: TTGCATCGACGACTATGAGC	GA	3	125–149	0.6139
	Ast17	DY-751	0.230	F: GGGCCTGTGACTGATTTTGT R: TGAAGGAGAAGAGGAAGAGGAA	TCT	16	145–193	0.3122
	Ast34	Cyanine 5	0.062	F: TGGGTTTTGAGAATTGAGCA R: TCAATAAAGGACCCTTAAAA GTCAA	GA	4	234–244	0.7033

Table 2 Variability of 18 polymorphic *Acipenser stellatus* microsatellite loci in two test panels of 20 farmed and 32 wild-caught individuals (N_A = number of alleles; N_{Ap} = number of private alleles; H_O = observed heterozygosity; H_E = expected heterozygosity; P_{HW} = exact P value of the Hardy-Weinberg probability test: $^*P < 0.05$, $^{**}P < 0.01$, n.s. non-significant)

Locus	Parameter	Farmed *A. stellatus*	Wild *A. stellatus*
Ast3	N_A	2	8
	N_{Ap}	0	6
	H_O	0.250	0.719
	H_E	0.481	0.627
	P_{HW}	n.s.	n.s.
Ast6	N_A	5	8
	N_{Ap}	0	3
	H_O	0.850	0.719
	H_E	0.754	0.785
	P_{HW}	n.s.	n.s.
Ast14	N_A	2	3
	N_{Ap}	0	1
	H_O	0.050	0.094
	H_E	0.050	0.092
	P_{HW}	n.s.	n.s.
Ast17	N_A	6	15
	N_{Ap}	1	10
	H_O	0.900	0.906
	H_E	0.767	0.892
	P_{HW}	n.s.	n.s.
Ast20	N_A	4	6
	N_{Ap}	0	2
	H_O	0.150	0.313
	H_E	0.191	0.337
	P_{HW}	n.s.	n.s.
Ast22	N_A	7	15
	N_{Ap}	0	8
	H_O	0.700	0.781
	H_E	0.656	0.922
	P_{HW}	n.s.	*
Ast25	N_A	5	17
	N_{Ap}	2	14
	H_O	0.950	0.656
	H_E	0.733	0.923
	P_{HW}	n.s.	**
Ast26	N_A	4	11
	N_{Ap}	0	7
	H_O	0.500	0.875
	H_E	0.594	0.837
	P_{HW}	**	n.s.
Ast30	N_A	5	34
	N_{Ap}	2	31
	H_O	0.400	0.969
	H_E	0.601	0.964
	P_{HW}	n.s.	**
Ast34	N_A	2	4
	N_{Ap}	0	2
	H_O	0.100	0.375
	H_E	0.097	0.455
	P_{HW}	n.s.	n.s.
Ast36	N_A	2	3
	N_{Ap}	0	1
	H_O	0.700	0.313
	H_E	0.467	0.395
	P_{HW}	*	n.s.
Ast39	N_A	2	3
	N_{Ap}	0	1
	H_O	0.150	0.437
	H_E	0.142	0.411
	P_{HW}	n.s.	n.s.
Ast43	N_A	3	5
	N_{Ap}	0	2
	H_O	0.800	0.531
	H_E	0.672	0.624
	P_{HW}	n.s.	n.s.
Ast44	N_A	2	3
	N_{Ap}	0	1
	H_O	0.600	0.500
	H_E	0.492	0.438
	P_{HW}	n.s.	n.s.
Ast48	N_A	2	3
	N_{Ap}	0	1
	H_O	0.600	0.125
	H_E	0.508	0.177
	P_{HW}	n.s.	*
Ast51	N_A	2	3
	N_{Ap}	0	1
	H_O	0.300	0.531
	H_E	0.492	0.458
	P_{HW}	n.s.	n.s.
Ast53	N_A	2	3
	N_{Ap}	0	1
	H_O	0.050	0.156
	H_E	0.050	0.148
	P_{HW}	n.s.	n.s.
Ast57	N_A	2	3
	N_{Ap}	0	1
	H_O	0.050	0.533
	H_E	0.224	0.569
	P_{HW}	*	*

of null alleles. However, only one locus (*Ast57*) deviated in both groups and should therefore be used with caution in

subsequent studies. On the other hand, the relatively small sample sizes of both groups might also be a reason for the observed deviations. The probability of identity for siblings at single microsatellite loci ranged from 0.3012 at locus *Ast30* to 0.9273 at locus *Ast14* (Table 1); the overall PIsib value was 8.73×10^{-6}. Genetic differentiation between farmed and wild stellate sturgeons was significant ($F_{ST} = 0.118$; $P < 0.05$). A high sensitivity of the 18 microsatellite loci was also demonstrated by self-classification: 51 out of 52 individuals (98%) were correctly identified; only one farmed stellate sturgeon was classified into the wild group.

In conclusion, the newly described 18 microsatellite loci provide sufficient variability and discriminatory power and are thus suitable to assess the genetic diversity and structure of the remaining wild populations of stellate sturgeon, to aid the development of both *in situ* and *ex situ* conservation measures, and to establish appropriate breeding schemes for supportive stocking programs.

Acknowledgments We thank Mr. Peter Groß, fish farm "Rhönforelle," Gersfeld, Germany, for generously providing access to the offspring of the *ex situ* stocks for sampling. Marian Paraschiv, Ştefan Honţ, and Marian Iani of the DDNI Sturgeon Research Group collected most of the tissue samples from the Danube River in Romania.

Funding This project was sponsored by the COFASP (Cooperation in Fisheries, Aquaculture and Seafood Processing) ERA-NET partners, which has received funding from the European Union's Seventh Framework Programme for Research, Technological Development and Demonstration under grant agreement no. 321553. Financial support was provided by the German Federal Ministry of Food and Agriculture (BMEL) through the Federal Office for Agriculture and Food (BLE), grant number 2814ERA01G, and the Romanian Executive Agency for Higher Education, Research, Development and Innovation Funding (UEFISCDI), contract number 26/2015.

Data availability DNA sequences of the 18 suitable microsatellite loci were submitted to GenBank (accession numbers KY496652-KY496669).

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ENVIRONMENTAL BIOTECHNOLOGY 13 (1) 2017, 11-17; doi:10.14799/ebms285

New microsatellite multiplex PCR sets for genetic studies of the sterlet sturgeon, *Acipenser ruthenus*

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Key words: *Acipenser ruthenus*, microsatellite, multiplex PCR, species conservation, sustainable fisheries

Received in May 2017. Published in December 2017.

ABSTRACT

Wild populations of the sterlet sturgeon, *Acipenser ruthenus*, are declining throughout their native ranges. In-depth knowledge of their genetic diversity and structure is urgently needed to enable the identification of management units for conservation purposes. Moreover, genetic markers are required to establish appropriate breeding schemes for supportive stocking programs and to monitor genetic changes in farmed stocks. Therefore, six species-specific, polymorphic microsatellite loci were isolated and arranged into five multiplex PCR sets together with nine loci from other sturgeon species. The diversity of these 15 microsatellites was examined in 67 sterlet individuals (20 farmed in Germany and 47 wild-caught in the Romanian part of the River Danube). The total number of alleles per locus ranged from 3 to 15 with an average of 7.20. The farmed sterlet sturgeon possessed 1 to 7 alleles per locus, with a mean of 3.13; the wild individuals were more variable, with 3 to 15 alleles per locus and a mean of 7.07. Observed heterozygosities ranged from 0 to 0.850 in the farmed individuals, and from 0.064 to 0.957 in the wild individuals. Indications of inbreeding were only found in the wild sterlet sturgeon (F_{IS}=0.062). The genetic differentiation of the two sterlet groups was significant (F_{ST}=0.1186). The high sensitivity and discriminatory power of the 15 loci was indicated by the very low overall probability of identity for siblings (PIsib=5.099x10^{-5}) and the high accuracy of self-classification (66 out of the 67 individuals (98.51%) were correctly identified). Thus, these newly developed multiplex PCR sets are a valuable genetic tool for identifying management units for species conservation, sustainable fisheries and aquaculture.

INTRODUCTION

The sterlet sturgeon (*Acipenser ruthenus* Linnaeus) is a relatively small potamodromous species that rarely reaches 1.2m in total length. It is native to tributaries of the northern Black Sea and Caspian Sea, and to Siberian rivers. While the sterlet is one of the few Eurasian sturgeon species that still has self-sustaining populations in most of its historic range, the population sizes have been drastically reduced, mostly due to hydroconstruction and overfishing (Gesner et al. 2010).

Key components of a responsible approach to stock enhancement programs have been identified and discussed by Blankenship and Leber (1995) and updated by Lorenzen et al. (2010). They include (among others) species management plans that include genetic objectives, genetic resource management to avoid deleterious genetic effects, and identification of released hatchery fish used for supportive stocking.

Supportive stocking measures or even re-establishment of sterlet populations are currently being attempted in the Danube, the Don, and the Kuban Rivers. In a variety of other river drainages, sterlet sturgeon is subject to recovery management, including the prohibition of directed fisheries and the establishment of *ex situ* stocks. The precautionary principle applies to management measures that use stocking as a recovery tool; this includes the identification and use of the indigenous populations in stocking measures. This approach

has been undermined in the Danube, as shown by the presence of Volga genotypes in the population, which originate from transplants and from escapees from aquaculture facilities in the Danube catchment area (Reinartz et al. 2011).

Since morphological differentiation is not a reliable method for distinguishing sterlet populations, genetic determination of the populations or sub-populations in question should be used (Dudu et al. 2011; Ludwig et al. 2009). Therefore, the aim of the present work was to develop multiplex PCR sets for reliable and time- and cost-efficient amplification of microsatellite loci in sterlet sturgeon, which could be used to identify management units via studies on population structure and differentiation, to establish breeding schemes for supportive stocking programs that maintain genetic diversity and integrity, and to monitor genetic changes in farmed populations (in particular, loss of genetic variability due to drift and inbreeding).

To the best of our knowledge, the six loci directly isolated from sterlet sturgeon genomic libraries are the first microsatellites described from this species; a search in NCBI GenBank (https://www.ncbi.nlm.nih.gov/genbank/ accesion date April, 21st 2017) revealed 305 entries for the pure species *Acipenser ruthenus* and nine entries for hybrids with *A. ruthenus* as one of the parental species. None of these records contained microsatellite DNA sequences.

MATERIALS AND METHODS

Twenty juvenile sterlet sturgeons of the same age were collected at the "Rhönforelle" fish farm, in Gersfeld, Germany. They were progeny of approximately 20 male and 20 female parents propagated artificially (personal communication, P. Groß, "Rhönforelle", Gersfeld). Fin clips were taken to isolate total genomic DNA using the DNeasy Blood & Tissue Kit (QIAGEN) according to the manufacturer's protocols. A pool of 10 DNA isolates was sent to GenoScreen, Lille, France (www.genoscreen.fr), where 1μg of the pooled DNA was used for the development of microsatellite libraries by 454 GS-FLX Titanium pyrosequencing of enriched DNA libraries as described in Malausa et al. (2011). Briefly, total DNA was mechanically fragmented and enriched for AG, AC, AAC, AAG, AGG, ACG, ACAT and ATCT repeat motifs. The enriched fragments were subsequently amplified. The PCR products were purified and quantified, and the GsFLX libraries were constructed following the manufacturer's protocols and sequenced on a GsFLX PTP. The bioinformatics program QDD (Meglećz et al. 2010) was used to analyze sequences. QDD performs all bioinformatics steps for treating raw sequences: removing adapters/vectors, detecting microsatellites, detecting redundancy/possible mobile element association, selecting sequences with target microsatellites, and designing primers by using BLAST (Altschul et al. 1990), Clustal W (Larkin et al. 2007), and Primer3 (Rozen and Skaletsky 2000). Out of 3942 sequences containing a microsatellite motif, 161 bioinformatically validated primer pairs were designed.

Based on our experience with isolation of microsatellite loci in tench, *Tinca tinca* (Kohlmann and Kersten 2006), and pike-perch, *Sander lucioperca* (Kohlmann and Kersten 2008), all sequences with validated primer pairs were ranked according to motif type (penta- > tetra- > tri- > di-nucleotide repeats), number of repeats (the higher the better), and PCR product size (>100bp), taking into consideration only sequences with perfect repeats. From this list, the 70 top-ranked primer pairs were selected for identification of suitable microsatellites (with consistent amplification, disomic pattern, ease of scoring, sufficient variability).

In addition to the microsatellite loci directly isolated from sterlet sturgeon as described above, nine microsatellites originating from other sturgeon species were included in the development of multiplex PCR sets. Locus *Spl-163* was isolated from shovelnose sturgeon (*Scaphirhynchus platorynchus*) subgenomic libraries enriched for microsatellites (McQuown et al. 2000), and subsequently shown to cross-amplify polymorphic loci in sterlet sturgeon by Fopp-Bayat and Furgała-Selezniow (2010). Loci *AfuG 41* and *AfuG 51* were isolated from lake sturgeon (*Acipenser fulvescens*) microsatellite-enriched genomic DNA (Welsh et al. 2003); locus *An20* was isolated from a partial genomic library of the Adriatic sturgeon (*A. naccarii*) enriched for GATA repeats (Zane et al. 2002); loci *Aox*D161 and *Aox*D165 were isolated from Atlantic sturgeon (*A. oxyrinchus*) microsatellite-enriched genomic libraries (Henderson-Arzapalo and King 2002); and all five of these loci were subsequently shown to cross-amplify polymorphic loci in sterlet sturgeon by Barmintseva and Mugue (2013). Loci *LS-19* (= *Afu 19*), *LS-39* (= *Afu-39*) and *LS-68* (= *Afu-68*) were isolated from a lake sturgeon (*A. fulvescens*) genomic library (May et al. 1997), and subsequently shown to cross-amplify polymorphic loci in sterlet sturgeon by Ludwig et al. (2001), Fopp-Bayat and Furgała-Selezniow (2010), and Dudu et al. (2013).

The initial procedure for selecting suitable loci was based on separate genotyping of single loci. However, since the final aim was to use time- and cost-saving multiplex PCR sets for future routine genotyping, protocols for microsatellite amplification were developed using PCR multiplex Kits (QIAGEN) and a peqSTAR 96X Universal Gradient thermocycler (Peqlab). After suitable loci had been identified, the software MultiPLX, version 2.1 (Kaplinski et al. 2005) was used to analyze PCR primer compatibility and automatically find the optimal multiplexing (grouping) solution. Three different dye labels (Cyanine 5, DY-751, Atto 680) were assigned to forward primers. Genotyping of microsatellite loci was performed on a CEQ 8000 8-capillary sequencer (Beckman Coulter) using the Fragment Analysis module of the GenomeLab™ GeXP Genetic Analysis System, version 10.2 (Beckman Coulter).

Microsatellite variability was initially examined in the 20 farmed individuals, but later, also examined in 47 wild sterlet sturgeons originating from the Romanian part of the River Danube. Young-of-the-year sterlet were captured in the Danube at km 123 using bottom drifting trammel nets with 18-20 mm mesh size during May-June in seven different years. Adult sterlet samples were taken from live adults captured for controlled

propagation on the Borcea branch by local professional fishermen. Prior to microsatellite genotyping, the species identity (i.e. that they were sterlet) and purity (i.e. that they were not sterlet hybrids) of all 67 individuals was confirmed using the nuclear DNA markers described by Havelka et al. (2017).

All microsatellite genotypes were examined with Micro-Checker software, version 2.2.3 (van Oosterhout et al. 2004) for scoring errors due to null alleles, stutter bands, and/or large allele dropout. Then, general characteristics of microsatellite loci variability (number of alleles, number of private alleles, observed and expected heterozygosity) were calculated, and tests for significance of deviations from Hardy-Weinberg equilibrium and linkage disequilibrium were performed using GENEPOP 4.0 (Rousset 2008). The discriminatory power of loci (probability of identity=PI) was estimated with GENECAP version 1.4 (Wilberg and Dreher 2004) applying the more conservative measure of PI for siblings, PIsib (Waits et al. 2001). Genetic differentiation between farmed and wild sterlet sturgeons was tested by calculating the F_{ST}-value using FSTAT, version 2.9.3.2 (Goudet 2002). FSTAT was also used to estimate the inbreeding coefficient F_{IS} for both groups of sterlet. Finally, the sensitivity of the 15 microsatellite loci was examined by self-classification of the 67 sterlet sturgeons using Bayesian criteria and the "Leave One Out" procedure of GeneClass software, version 1.0.02 (Cornuet et al. 1999).

RESULTS AND DISCUSSION

Table 1. Characteristics of 15 polymorphic microsatellite loci tested in a total of 67 *Acipenser ruthenus* individuals.

PCR multiplex set (annealing temperature)	Locus name / GenBank accession no.	Forward primer dye label	Primer concentration (μM)	Primer sequence (5'- 3')	Repeat type	Total number of alleles	Allele size range (bp)
1 (57°C)	*Spl-163*[a] *AF276205*	Cyanine 5	0.033	F: TGCTTGTAAACTGCCCCACT R: CCACATGCAGTTTGAGCTGC	GATA	10	172-220
	Aru26 MF100775	DY-751	0.180	F: AAAGCAACAACTCCACCAGG R: TGCCTTGTCTACTGTCCGAA	CT	3	159-163
	AfuG 41[b] *AF529463*	Atto 680	0.210	F: TGACGCACAGTAGTATTATTTATG R: TGATGTTTGCTGAGGCTTTTC	(GATA)TA(GATA)	14	193-261
2 (57°C)	*An20*[c] *AY144618*	Cyanine 5	0.038	F: AATAACAATCATTACATGAGGCT R: TGGTCAGTTGTTTTTTTATTGAT	(ATCT)(TG)	11	152-178
	Aru12 MF100771	DY-751	0.170	F: AAATAGCATGTTCCCCAGCA R: TCCATTGCACTTTTCCTTCTTT	TAG	3	172-178
	LS-39[d] *U72734*	Atto 680	0.190	F: TTCTGAAGTTCACACATTG R: ATGGAGCATTATTGGAAGG	GTT	4	117-132
3 (57°C)	*AfuG 51*[b] *AF529467*	Cyanine 5	0.041	F: ATAATAATGAGCGTGCTTTCTGTT R: ATTCCGCTTGCGACTTATTTA	(AAAC)(AC)(AAAC)	5	223-247
	AoxD165[e] *AY093640*	DY-751	0.180	F: TTTGACAGCTCCTAAGTGATACC R: AAAGCCCTACAACAAATGTCAC	(CTAT)CTAC(CTAT)	10	170-206
	LS-19[d] *U72730*	Atto 680	0.160	F: CATCTTAGCCGTCTGTGGTAC R: CAGGTCCCTAATACAATGGC	TTG	3	138-144
4 (60°C)	*LS-68*[d] *U72739*	Cyanine 5	0.044	F: TTATTGCATGGTGTAGCTAAAC R: AGCCCAACACAGACAATATC	GATA	15	174-238
	Aru13 MF100772	DY-751	0.190	F: TCCACTTTATTCCGTTGTGG R: AGACCGGAATCAAACCCAG	GTT	13	87-135
	AoxD161[e] *AY093639*	Atto 680	0.180	F: GTTTGAAATGATTGAGAAAATGC R: TGAGACAGACACTCTAGTTAAACAGC	CTAT	6	106-130
5 (60°C)	*Aru19 MF100774*	Cyanine 5	0.038	F: GCGTGGTGTAAGTGAACCCT R: CTTCAATTGTGCTTGGCTCA	GA	4	159-193
	Aru50 MF100780	DY-751	0.210	F: TGGAAACCAAATTAATTCACAAAA R: TGGGATCCTCTGTAGAACAGTCT	AG	3	123-129
	Aru18 MF100773	Atto 680	0.180	F: CCTGGAACACGTCCAGTTTT R: TGGGTGAATGTTTTGGTGTG	TC	4	135-145

References for microsatellite locus names, GenBank accession numbers, primer sequences and repeat type information: [a] McQuown et al. (2000), [b] Welsh et al. (2003), [c] Zane et al. (2002), [d] May et al. (1997), [e] Henderson-Arzapalo and King (2002).

Out of the 70 sterletsturgeon PCR primer pairs chosen for testing, six amplified microsatellite loci matched our selection criteria, in particular, consistent amplification, disomic pattern, and sufficient variability. These six loci were combined with the nine loci originating from other sturgeon species into five multiplex PCR sets (Table 1). The optimized PCR reaction mixes consisted of 5.0μL of master mix and 1.5μL Q-solution (QIAGEN), 1.0μL DNA isolate, primers at the concentrations given in Table 1, and PCR-grade water up to a final volume of 10.0μL. The PCR program, based on QIAGEN recommendations, included initial denaturation at 95°C for 15min, followed by 30 cycles of denaturation at 94°C for 30s, annealing at 57°C (multiplex sets 1, 2 and 3) or 60°C (multiplex sets 4 and 5) for 90s, and extension at 72°C for 60s. Final extension at 60°C lasted for 30min.

Tests with Micro-Checker software did not reveal any evidence of scoring errors due to stutter bands or large allele dropout. Moreover, no indications of null alleles were found at any of the 15 loci of the 20 farmed individuals. In contrast, homozygote excess was observed at four loci (*Spl-163*, *LS-68*, *Aru19*, and *Aru50*) in the 47 wild sterlet sturgeons, suggesting that null alleles might be present at these loci. The total number of alleles per locus ranged from 3 to 15 (Table 1), with an average of 7.20. The farmed sterlet sturgeon possessed 1 to 7 alleles per locus, with a mean number of 3.13; the wild individuals were more variable, and had 3 to 15 alleles per locus, with a mean number of 7.07 (Table 2). Consequently, private alleles were almost exclusively found in the wild sterlet sturgeons (Table 2). Linkage disequilibrium tests for each locus pair across both groups of sterlet revealed that three of the 105 possible loci pair combinations gave significant *P*-values: *Spl-163* and *LS-68* (P=0.003), *LS-68* and *Aru13* (P=0.018), and *LS-19* and *Aru19* (P=0.045). The observed heterozygosities ranged from zero at loci *Aru18*, *Aru26*, and *Aru50*, to 0.850 at loci *Aru12* and *Spl-163* in the farmed individuals, and from 0.064 at locus *Aru50* to 0.957 at locus *Aru12* in the wild individuals (Table 2). Significant (P<0.05) to highly significant (P<0.01) deviations from Hardy-Weinberg equilibrium (P_{HW}) were found at three loci in the farmed sturgeons and six loci in the wild sterlet sturgeons (Table 2). However, indications of inbreeding (F_{IS}) were only found in the wild sterlet sturgeon, with an F_{IS}-value of 0.062 (the corresponding value for the farmed sterlet was negative: -0.071). With regard to the farmed sterlet, the main reason for the deviations from Hardy-Weinberg equilibrium might be the relatively small sample size. In the 47 wild sterlet sturgeons, the possible presence of null alleles at four microsatellite loci, and the uneven representation of sampling years in the data set (the number of individuals per year ranged from 1 to 16) could have contributed to deviations from equilibrium. This will be investigated in more detail in future studies with larger numbers of wild individuals.

Table 2. Variability of 15 polymorphic microsatellite loci in two test panels of 20 farmed and 47 wild-caught *Acipenser ruthenus* (N_A=number of alleles; N_{Ap}=number of private alleles; H_O=observed heterozygosity; H_E=expected heterozygosity; P_{HW}=results of the Hardy-Weinberg probability test: *P<0.05, **P<0.01, n.s.=non-significant, n.a.=not applicable).

Locus	Parameter	Farmed *A. ruthenus*	Wild *A. ruthenus*	Probability of identity for siblings (PIsib)
Spl-163	N_A	7	8	0.3323
	N_{Ap}	2	3	
	H_O	0.850	0.638	
	H_E	0.835	0.826	
	P_{HW}	n.s.	*	
Aru26	N_A	1	3	0.9291
	N_{Ap}	0	2	
	H_O	0	0.106	
	H_E	0	0.103	
	P_{HW}	n.a.	n.s.	
AfuG 41	N_A	5	14	0.3458
	N_{Ap}	0	9	
	H_O	0.800	0.872	
	H_E	0.737	0.868	
	P_{HW}	n.s.	n.s.	
An20	N_A	3	11	0.3407
	N_{Ap}	0	8	
	H_O	0.250	0.830	
	H_E	0.232	0.875	
	P_{HW}	n.s.	*	

Aru12	N_A	2	3	0.5924
	N_{Ap}	0	1	
	H_O	0.700	0.957	
	H_E	0.467	0.515	
	P_{HW}	*	**	
LS-39	N_A	2	4	0.5694
	N_{Ap}	0	2	
	H_O	0.300	0.511	
	H_E	0.385	0.556	
	P_{HW}	n.s.	n.s.	
AfuG 51	N_A	2	5	0.7575
	N_{Ap}	0	3	
	H_O	0.450	0.149	
	H_E	0.450	0.143	
	P_{HW}	n.s.	n.s.	
AoxD165	N_A	4	10	0.3702
	N_{Ap}	0	6	
	H_O	0.550	0.745	
	H_E	0.635	0.813	
	P_{HW}	n.s.	n.s.	
LS-19	N_A	2	3	0.7150
	N_{Ap}	0	1	
	H_O	0.200	0.383	
	H_E	0.185	0.375	
	P_{HW}	n.s.	n.s.	
LS-68	N_A	7	15	0.3170
	N_{Ap}	0	8	
	H_O	0.650	0.617	
	H_E	0.760	0.899	
	P_{HW}	*	**	
Aru13	N_A	3	13	0.3586
	N_{Ap}	0	10	
	H_O	0.850	0.830	
	H_E	0.581	0.858	
	P_{HW}	**	n.s.	
AoxD161	N_A	4	6	0.4600
	N_{Ap}	0	2	
	H_O	0.800	0.574	
	H_E	0.658	0.618	
	P_{HW}	n.s.	n.s.	
Aru19	N_A	3	4	0.5735
	N_{Ap}	0	1	
	H_O	0.550	0.319	
	H_E	0.578	0.445	
	P_{HW}	n.s.	*	
Aru50	N_A	1	3	0.8331
	N_{Ap}	0	2	
	H_O	0	0.064	
	H_E	0	0.246	
	P_{HW}	n.a.	**	
Aru18	N_A	1	4	0.8298
	N_{Ap}	0	3	
	H_O	0	0.277	
	H_E	0	0.251	
	P_{HW}	n.a.	n.s.	

The probability of identity for siblings (PIsib) at single microsatellite loci ranged from 0.3170 at locus *LS-68* to 0.9291 at locus *Aru26* (Table 2); the overall PIsib value was very low: 5.099×10^{-5}. Genetic differentiation between farmed and wild sterlet sturgeons was significant ($F_{ST}=0.1186$; $P<0.05$). The accuracy of classification with the 15 microsatellite loci was also demonstrated by the fact that 66 out of the 67 individuals (=98.51%) were correctly identified using these loci; only one wild sterlet sturgeon was assigned to the farmed group.

In conclusion, these five multiplex PCR sets, which consist of six microsatellite loci isolated from sterlet sturgeon and nine microsatellite loci from other sturgeon species, provide sufficient variability and should thus be suitable for assessing the genetic diversity and structure of the remaining wild populations, for aiding the development of both *in situ* and *ex situ* conservation measures, for establishing appropriate breeding schemes for supportive stocking programs, and for monitoring genetic changes in farmed strains used for caviar and meat production.

Data availability

The DNA sequences of the six newly isolated *Aru* microsatellite loci were deposited in NCBI GenBank (for accession numbers see Table 1).

Funding

This project was sponsored by the COFASP ERA-NET partners, which has received funding from the European Union's Seventh Framework Programme for research, technological development and demonstration under grant agreement no. 321553. Financial support was provided by the German Federal Ministry of Food and Agriculture (BMEL) through the Federal Office for Agriculture and Food (BLE), grant number 2814ERA01G; and by the Romanian Executive Agency for Higher Education, Research, Development and Innovation Funding (UEFISCDI), contract number 26/2015.

ACKNOWLEDGEMENTS

We thank Mr. Peter Groß, "Rhönforelle" fish farm, Gersfeld, Germany for generously providing access to the offspring of the *ex situ* stocks for sampling. Marian Paraschiv, Ștefan Honț and Marian Iani of the DDNI Sturgeon Research Group collected most of the tissue samples from the Danube River in Romania.

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Aquaculture International (2018) 26:1365–1376
https://doi.org/10.1007/s10499-018-0290-y

Validation of 12 species-specific, tetrasomic microsatellite loci from the Russian sturgeon, *Acipenser gueldenstaedtii*, for genetic broodstock management

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Received: 20 February 2018 / Accepted: 26 July 2018 / Published online: 2 August 2018

Abstract

The Russian sturgeon, *Acipenser gueldenstaedtii*, is a critically endangered fish species. Hatcheries are operated in several countries within its natural range to produce stocking material for release into the wild and also for aquaculture purposes (caviar and meat production). An appropriate genetic broodstock management (plan or strategy) is required to avoid negative effects, e.g., admixture and hybridization of genetically differing stocks or loss of genetic variability due to inbreeding and genetic drift. Therefore, 11 tetrasomic microsatellite loci were newly isolated from the Russian sturgeon genome and arranged together with an already known locus into four multiplex PCR sets. These microsatellites were used to characterize three groups of hatchery juveniles from Germany (aquaculture production), Turkey, and Romania (production of stocking material) as well as a group of wild-caught adults from the Danube River, Romania. Based on the variability within groups, measured by the mean number of alleles per locus and expected heterozygosity, and the differentiation between groups, measured by Nei's G_{ST} and genetic distance D, the ability of the 12 loci to detect unwanted reductions in genetic variability within hatchery juveniles and to differentiate between groups could be demonstrated. This set of loci can also be used to identify those pairs of spawners that transmit the highest possible genetic variability to the next generation.

Keywords *Acipenser gueldenstaedtii* · Broodstock · Genetic diversity · Hatchery · Microsatellite · Ploidy level · Russian sturgeon

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Introduction

The Russian sturgeon (*Acipenser gueldenstaedtii* Brandt & Ratzeburg, 1833) is a species of the family Acipenseridae native to the Caspian, Black, and Azov Sea basins. However, aquaculture has resulted in intentional and accidental introductions throughout Europe (Gesner et al. 2010). Originally, the species had freshwater as well as anadromous populations. Freshwater populations existed in the Danube and Volga Rivers—but both are extinct now (Gesner et al. 2010). Also, the anadromous spawning populations in the Azov Sea were lost and only introduced (stocked) individuals remained. Currently, native anadromous spawning populations are only known from the Caspian and Black Sea tributaries; but even these populations have undergone major declines resulting in no confirmed natural reproduction of the Danube River populations over the last 5 years (Suciu, unpubl. data). Consequently, the Russian sturgeon was classified as "critically endangered" in the IUCN Red List of Threatened Species (Gesner et al. 2010). Gesner et al. (2010) also listed the major threats to the species: loss of spawning sites due to dam construction (e.g., 70% of spawning grounds in the Caspian Sea basin since the 1950s), poaching and illegal fishing, which appears to be increasing, and high levels of pollution (from oil and industrial waste), in both the Black and Caspian Sea basins. Genetic pollution is also a potential threat as stocks are moved to different locations (e.g., Caspian Sea stocks moved to the Azov Sea). To compensate the dramatic losses in individual numbers, restocking measures are ongoing. However, stocks are continuing to decline.

With the ever increasing importance of artificial hatchery reproduction both for aquaculture purposes and the production of stocking material, an appropriate broodstock management has to be implemented. From the genetic point of view, the final goal must be to preserve the original genetic structures and variabilities to a degree that avoids (i) the disruption of local adaptations caused by the admixture and hybridization of genetically differing stocks, and (ii) inbreeding, which may result from the small number of breeders usually propagated in hatcheries, in particular if the species is rare. On the other hand, the genetic management should allow genetic change (i.e., natural selection) from pressures that occur in the wild (after release of the stocked fish), but not from pressures in the hatchery. This goal is related to the concept of integrated hatchery programs and conservation hatcheries as defined by Trushenski et al. (2015). To meet these requirements, genetic markers are urgently needed—and have to be developed if not already available—to implement breeding plans for the ex situ measures and for stocking.

Based on the number of chromosomes (250 ± 8) (Vasil'ev 1985; Vlasenko et al. 1989; both cited in Vasil'ev et al. 2010) and the allele banding patterns of microsatellite loci (Havelka et al. 2013; Rajkov et al. 2014), the Russian sturgeon is considered as an octaploid species at the evolutionary scale, which indicates the maximum ploidy level achieved, and a tetraploid one at the recent scale, which indicates the current functional ploidy level (Rajkov et al. 2014). Rajkov et al. (2014) could identify only one out of 20 microsatellite loci (= 5%) examined as disomic. Among the eight loci newly isolated from Russian sturgeon in their study, three loci (*Ag09*, *Ag28*, and *Ag49*) expressed the tetrasomic pattern expected for a tetraploid species, four loci (*Ag01*, *Ag12*, *Ag14*, and *Ag22*) displayed higher than tetrasomic patterns, and one locus (*Ag18*) was the disomic one mentioned above. This variation in microsatellite loci expression patterns of Russian sturgeon shows that the process of functional genome reduction is still ongoing in species with ~ 250 chromosomes (Ludwig et al. 2001).

Microsatellite loci are popular and efficient DNA markers widely applied in aquaculture genetics (Liu and Cordes 2004). However, they have limitations in the light of the present

development of sequencing technologies (Putman and Carbone 2014) that are underlined by their utilization in polyploid organisms (Dufresne et al. 2014; Meirmans et al. 2018). Although microsatellites might be displaced by single nucleotide polymorphisms (SNPs) for sturgeon conservation in future (Ogden et al. 2013), their suitability for the management of a captive broodstock of another tetraploid sturgeon species, the critically endangered Adriatic sturgeon *Acipenser naccarii*, could clearly be demonstrated (Congiu et al. 2011).

A search in NCBI GenBank (accession date: 13 July 2017) for DNA sequence records of the species *Acipenser gueldenstaedtii* revealed 423 entries. However, there were no other microsatellite loci deposited than the eight described in Rajkov et al. (2014). Therefore, the aim of the present study was doubled: (i) to examine the variability of the three tetrasomic loci described by Rajkov et al. (2014) in a larger number of individuals and populations, and (ii) to isolate additional microsatellites from the Russian sturgeon genome to obtain a sufficient number of markers for improved broodstock management and the characterization of population structures.

Material and methods

Development of new microsatellite loci from the Russian sturgeon genome

Fin clips were collected from 20 juvenile Russian sturgeons at the "Rhönforelle" fish farm in Gersfeld, Germany, to isolate total genomic DNA using the DNeasy Blood & Tissue Kit (QIAGEN) according to manufacturer's protocols. A pool of ten DNA isolates was sent to GenoScreen, Lille, France (www.genoscreen.fr), where 1 μg of the pooled DNA was used for the development of microsatellite libraries through 454 GS-FLX Titanium pyrosequencing as described in Malausa et al. (2011). The bioinformatics program QDD (Meglécz et al. 2010) was used to analyze sequences. QDD performs all steps from raw sequences until obtaining PCR primers: removing adapters/vectors, detecting microsatellites, detecting redundancy and possible mobile element association, selecting sequences with target microsatellites, and designing primers by using the BLAST (Altschul et al. 1990), Clustal W (Larkin et al. 2007), and Primer3 (Rozen and Skaletsky 2000) programs. Among 5752 sequences containing a microsatellite motif, 198 bioinformatically validated primer pairs were designed.

All sequences with validated primer pairs were ranked according to motif type (penta- > tetra- > tri- > di-nucleotide repeats), number of repeats (the higher the better), and PCR product size (> 100 bp) considering only sequences with perfect repeats. From this list, the 60 top-ranking primer pairs were selected for the identification of suitable microsatellites (consistent amplification, ease to score, sufficient variability). Amplification protocols were developed for future use of PCR multiplex kits (QIAGEN) and a peqSTAR 96X Universal Gradient thermocycler (Peqlab). PCR primers were multiplexed (grouped) by the software MultiPLX, version 2.1 (Kaplinski et al. 2005). Three different dye labels (BMN-6, Cyanine 5, and DY-751) were assigned to forward primers. Genotyping of microsatellite loci was performed on an eight-capillary sequencer CEQ 8000 (Beckman Coulter) using the Fragment Analysis module of the GenomeLab™ GeXP Genetic Analysis System, version 10.2 (Beckman Coulter).

The microsatellite variability was initially examined in the 20 juveniles from the German hatchery (broodstock and progenies kept for aquaculture purposes) but later on extended to 20 juveniles from the Romanian hatchery of Isaccea (F1 half-sibs of wild spawners used for restocking into the Danube River), 22 juveniles from a Turkish hatchery (F1 progeny of wild

females and hatchery males used for enhancement of natural resources) and 11 wild-caught adults from the Romanian part of the Danube River. Due to the scarcity of disomic loci in Russian sturgeon, the focus of the study was to identify suitable tetrasomic loci showing consistent amplification and sufficient polymorphism.

Data analysis

The difficulty of analyzing codominant tetrasomic microsatellite loci of tetraploid species is to identify the true genotypes for partial heterozygotes. For instance, the genotype ABBC produces the microsatellite phenotype ABC, and cannot be reliably differentiated from genotypes AABC or ABCC. Jenneckens et al. (2001) suggested to estimate the number of allele copies on the basis of peak heights and areas in the electropherograms as exemplified by the microsatellite locus *LS-39* in the Siberian sturgeon *Acipenser baerii*. However, this approach seems not to be fully reliable: Congiu et al. (2011) found that the four peaks of a complete heterozygous genotype did not always show the expected same height in Adriatic sturgeon, and the same discrepancy was observed in the present study of Russian sturgeon (data not shown). Instead, Congiu et al. (2011) proposed a band sharing approach for which microsatellite data were considered as presence/absence of bands, disregarding the number of alleles present in each individual. Another and probably more convenient solution of the problem is provided by software specifically designed for the analyses of (allo-)tetraploid microsatellite loci: TETRASAT (Markwith et al. 2006), TETRA (Liao et al. 2008), and ATETRA (Van Puyvelde et al. 2010). Van Puyvelde et al. (2010) compared the three programs and concluded that TETRASAT and ATETRA have a comparable precision but are both more precise than TETRA. Since ATETRA is able to calculate more genetic variables and can handle an infinite number of partial heterozygotes, this program was chosen for the present study.

ATETRA was used with default parameters for Monte-Carlo simulations to calculate (i) the Hardy-Weinberg expected heterozygosity or Nei's genetic diversity H_E (Nei 1987) as well as values corrected for sample size $H_{E,c}$, (ii) the populational differentiation or Nei's G_{ST} (Nei 1973), and (iii) Nei's genetic distance D (Nei 1972, 1978). The matrix of pairwise genetic distances between the four Russian sturgeon groups was then used to construct a neighbor-joining tree (Saitou and Nei 1987) with MEGA5 (Tamura et al. 2011).

Data availability The DNA sequences of the 11 newly isolated *Agu* microsatellite loci were deposited in NCBI GenBank (for accession numbers see Table 1).

Results and discussion

Out of the three tetrasomic microsatellites chosen from Rajkov et al. (2014) for testing two loci displayed pentasomy (*Ag09* in two out of 40 samples, and *Ag28* in two out of eight samples) and were therefore discarded from further use. Only one locus (*Ag49*) displayed the expected tetrasomic pattern consistently and was included in further analyses. Out of the 60 new Russian sturgeon PCR primer pairs chosen for testing, 11 amplified microsatellite loci matched our selection criteria, in particular consistent amplification, tetrasomic pattern, and sufficient variability. These 11 loci were combined with locus *Ag49* into four

Table 1 Characteristics of the 12 polymorphic, tetrasomic microsatellite loci tested in a total number of 73 Russian sturgeons

PCR multiplex set (annealing temperature)	Locus name/GenBank accession no.	Forward primer dye label	Primer concentration (μM)	Primer sequence (5′-3′)	Repeat type	Total number of alleles	Allele size range (bp)
1 (63 °C)	*Agu56*/MG956817	BMN-6	0.200	F: TGATTGGTAATAATGCCTGCC R: TGTCACAGGGGACAGATTCA	TC	5	148–158
	Agu51/MG956815	DY-751	0.180	F: TACATCCACAGCACCTTCCA R: CCAGAGCTGGCTGTATGTGA	CT	5	141–159
	Agu38/MG956812	BMN-6	0.175	F: ACTGGGGTTGAAGGACAGTG R: TCCGTCTCATGTCCAAGGTA	GA	4	108–114
2 (63 °C)	*Agu54*/MG956816	Cyanine 5	0.055	F: GGAGCCAGTATCCCCTCAAT R: CTTCGCACGCAACTTAACAA	TG	5	188–214
	Agu36/MG956810	DY-751	0.195	F: GCAAACTGGGCTAGAACCTG R: TCCCCTCTCCTCTTCTCCTC	AG	11	104–128
	Agu37/MG956811	BMN-6	0.195	F: ACATGGTAGCAAAATCCCAA R: CAGCAAGCTTAGATGCATGG	CT	5	128–136
3 (63 °C)	*Ag49*[a]/HG931711	Cyanine 5	0.075	F: TGTTATCTGCTCTGATATTGATTCG R: CGTTTTAAAGTTTGAACGGCA	TTC	7	198–219
	Agu15/MG956808	DY-751	0.500	F: CGCAGCACAACTGCACAT R: CTGCCAGTGCGGTATCTACA	AGG	13	120–165
	Agu59/MG956818	BMN-6	0.170	F: TCGAAACCATAAGCGTTGAA R: TGACACTTGTGAAGCAACAGC	TG	5	210–280
4 (57 °C)	*Agu34*/MG956809	BMN-6	0.150	F: CGCAGCATGTGAAGGTTTTA R: CTCACAAACTTTGGATGCCT	GA	6	116–130
	Agu46/MG956814	DY-751	0.160	F: TGTACTGGAGTGTTTTTGAAAAGG R: CATTTGTTGGATACATTCAGCTTT	TC	5	103–113
	Agu41/MG956813	BMN-6	0.180	F: AAGACAAACAGTGGCCCAAC R: CAATGGCAGGTGCTACTGAA	AG	14	178–218

[a] Locus name, Genbank accession number, primer sequences, and repeat type information: supplementary Table S1 of Rajkov et al. (2014)

multiplex PCR sets (Table 1). Optimized PCR reaction mixes consisted of 5.0 μl of master mix and 1.5 μl Q-solution (QIAGEN), 1.0 μl DNA isolate, primers with concentrations as stated in Table 1, and PCR-grade water up to a final volume of 10.0 μl. The PCR program based on QIAGEN recommendations included an initial denaturation at 95 °C for 15 min followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C (multiplex set 4), or 63 °C (multiplex sets 1 to 3) for 90 s, and extension at 72 °C for 60 s. A final extension at 60 °C lasted for 30 min.

The observed total number of alleles per locus ranged from 4 to 14 (Table 1) with an average of 7.08. The hatchery juveniles possessed a mean number of alleles per locus ranging from 3.67 to 4.25; the wild-caught adults were more variable with a mean number of 5.67 (Table 2). However, the differences in mean number of alleles per locus were only significant

Table 2 Variability of the 12 tetrasomic microsatellite loci in the four Russian sturgeon groups (*A* number of alleles, H_E expected heterozygosity, $H_{E,c}$ expected heterozygosity, corrected for sample size)

Locus	Parameter	German hatchery	Romanian hatchery	Turkish hatchery	Romanian wild-caught
Agu56	*A*	3	4	1	5
	H_E	0.626	0.675	0.000	0.570
	$H_{E,c}$	0.634	0.684	0.000	0.583
Agu51	*A*	3	2	3	3
	H_E	0.429	0.496	0.488	0.507
	$H_{E,c}$	0.434	0.502	0.494	0.519
Agu38	*A*	3	2	3	4
	H_E	0.494	0.095	0.515	0.644
	$H_{E,c}$	0.500	0.096	0.521	0.659
Agu54	*A*	4	3	4	4
	H_E	0.746	0.542	0.733	0.669
	$H_{E,c}$	0.755	0.549	0.742	0.685
Agu36	*A*	6	4	4	7
	H_E	0.754	0.603	0.394	0.768
	$H_{E,c}$	0.764	0.611	0.399	0.786
Agu37	*A*	5	4	4	5
	H_E	0.755	0.380	0.566	0.698
	$H_{E,c}$	0.764	0.385	0.572	0.715
Ag49	*A*	3	6	6	7
	H_E	0.649	0.798	0.778	0.763
	$H_{E,c}$	0.657	0.808	0.787	0.780
Agu15	*A*	7	7	5	8
	H_E	0.700	0.773	0.600	0.771
	$H_{E,c}$	0.709	0.783	0.607	0.789
Agu59	*A*	2	2	2	5
	H_E	0.476	0.495	0.495	0.631
	$H_{E,c}$	0.483	0.501	0.501	0.646
Agu34	*A*	5	3	4	4
	H_E	0.745	0.512	0.664	0.676
	$H_{E,c}$	0.754	0.519	0.672	0.692
Agu46	*A*	4	2	4	5
	H_E	0.647	0.179	0.697	0.685
	$H_{E,c}$	0.655	0.181	0.705	0.701
Agu41	*A*	6	5	7	11
	H_E	0.758	0.674	0.830	0.852
	$H_{E,c}$	0.767	0.682	0.840	0.872
Mean values	*A*	4.25	3.67	3.92	5.67
	H_E	0.648	0.519	0.563	0.686
	$H_{E,c}$	0.656	0.525	0.570	0.702

Table 3 Populational differentiation G_{ST} (Nei 1973) (above diagonal) and genetic distance D (Nei 1972, 1978) (below diagonal) between the four groups of Russian sturgeon

	German hatchery	Romanian hatchery	Turkish hatchery	Romanian wild-caught
German hatchery		0.113	0.074	0.033
Romanian hatchery	0.412		0.143	0.066
Turkish hatchery	0.263	0.491		0.062
Romanian wild-caught	0.150	0.202	0.220	

between the wild-caught adults and the hatchery juveniles from Romania ($P = 0.02$; two-sided t test) and Turkey ($P = 0.04$; two-sided t test), respectively. Mean expected heterozygosities H_E were also lower in the hatchery juveniles, ranging from 0.519 to 0.648, than in the wild-caught adults with a value of 0.686 (Table 2). A significant difference was only found between the two Romanian groups ($P = 0.02$; two-sided t test). The correction for sample size resulted in only slight changes; again only the difference between the two Romanian groups was statistically significant ($P = 0.02$; two-sided t test).

Pairwise population differentiation was lowest between German hatchery juveniles and Romanian wild-caught adults ($G_{ST} = 0.033$) and highest between Romanian and Turkish hatchery juveniles ($G_{ST} = 0.143$) (Table 3). Accordingly, the lowest genetic distance was observed between German hatchery juveniles and Romanian wild-caught adults ($D = 0.150$) and the highest between Romanian and Turkish hatchery juveniles ($D = 0.491$) (Table 3). The neighbor-joining algorithm does not simply join the two groups displaying the lowest genetic distance (i.e., German hatchery juveniles and Romanian wild-caught adults) but also takes into consideration the genetic distances to all other groups. Hence, on the neighbor-joining tree (Fig. 1) the German and Turkish hatchery juveniles ($D = 0.263$) were placed together into one group separated from a Romanian group consisting of the hatchery juveniles and wild-caught adults ($D = 0.202$).

Comparable studies on Russian sturgeon or other tetraploid sturgeon species are still relatively rare. Most of them dealt either with the isolation and characterization of new microsatellite loci or their use as nuclear markers for sturgeon species and hybrid identification and to examine genome duplication events (Table 4). Comparisons of general microsatellite variability might be biased by ploidy level and sample size. However, if only tetrasomic loci were considered then the ranges of numbers of alleles per locus are rather similar across different loci and sturgeon species (Table 4): Russian sturgeon—4-14 (present study), 6–16 (Ludwig et al. 2001), 7–19 (Havelka et al. 2013); Adriatic sturgeon—8-16 (Congiu et al. 2011); lake sturgeon—8-10 (McQuown et al. 2003); Siberian sturgeon—11-18 (Barmintseva and Mugue 2017). Heterozygosity estimates are only available for disomic loci of the lake sturgeon (Table 4). Nevertheless, they are also at similar levels as the expected heterozygosities

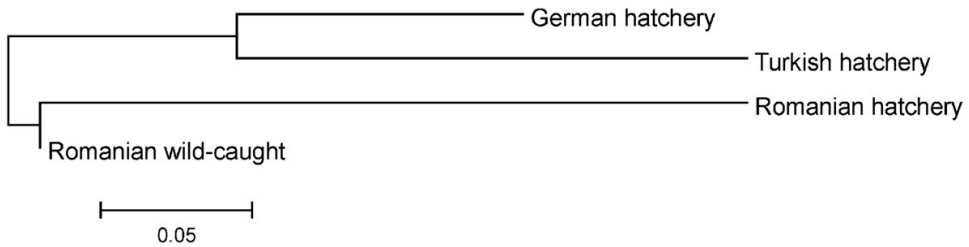

Fig. 1 Neighbor-joining tree of the four Russian sturgeon groups based on genetic distance D (Nei 1972, 1978)

Table 4 Use of microsatellite loci in tetraploid sturgeon species (n.d. no data reported)

Sturgeon species	Sample size	Origin of microsatellite loci	Number of loci (allelic pattern)	Number of alleles per locus	Heterozygosity (H_O observed, H_E expected)	Purpose of the study	Reference
Russian sturgeon (*A. gueldenstaedtii*)	99	Lake sturgeon (*A. fulvescens*)	5 (4 tetrasomic, 1 octosomic)	6–16 (tetrasomic loci); 28 (octosomic locus)	n.d.	Examination of genome duplications in sturgeons	Ludwig et al. (2001)
	16	Lake sturgeon (*A. fulvescens*), Atlantic sturgeon (*A. oxyrinchus*), and Adriatic sturgeon (*A. naccarii*)	8 (some tetrasomic)	1–8	n.d.	Nuclear markers for detection of Danube sturgeons hybridization	Dudu et al. (2011)
	35	Lake sturgeon (*A. fulvescens*), Atlantic sturgeon (*A. oxyrinchus*), white sturgeon (*A. transmontanus*), and shovelnose sturgeon (*Scaphirhynchus platorynchus*)	11 (7 tetrasomic, 4 octosomic)	7–19 (tetrasomic loci); 19–26 (octosomic loci)	n.d.	Examination of genome duplications in sturgeons	Havelka et al. (2013)
Adriatic sturgeon (*A. naccarii*)	20	Adriatic sturgeon (*A. naccarii*)	7 (higher than disomic)	3–19	n.d.	Isolation and characterization of microsatellites	Zane et al. (2002)
	42	Adriatic sturgeon (*A. naccarii*), lake sturgeon (*A. fulvescens*), Atlantic sturgeon (*A. oxyrinchus*), and shovelnose sturgeon (*Scaphirhynchus platorynchus*)	24 (12 tetrasomic, 4 disomic, 7 with max. 3 alleles, 1 with max. 7 alleles)	2–8 (disomic loci); 8–16 (tetrasomic loci)	n.d.	Managing polyploidy in ex situ conservation genetics	Congiu et al. (2011)
	20	Adriatic sturgeon (*A. naccarii*)	24 from genomic library, 33 from transcriptomic	2–7; 2–16	n.d.; n.d.	Isolation and characterization of microsatellites	Boscari et al. (2015)

Table 4 (continued)

Sturgeon species	Sample size	Origin of microsatellite loci	Number of loci (allelic pattern)	Number of alleles per locus	Heterozygosity (H_O observed, H_E expected)	Purpose of the study	Reference
			library (some higher than tetrasomic)			from the genome and transcriptome	
Lake sturgeon (*A. fulvescens*)	210 (15–40) from 7 locations	Lake sturgeon (*A. fulvescens*), Atlantic sturgeon (*A. oxyrinchus*), and shovelnose sturgeon (*Scaphirhynchus platorynchus*)	7 (4 disomic, 3 presumptive tetrasomic)	5–12 (disomic loci); 8–10 (presumptive tetrasomic loci)	H_O = 0.46–0.66 (based on the 4 disomic loci)	Genetic comparison of populations	McQuown et al. (2003)
	943 (1–136) from 27 locations	Lake sturgeon (*A. fulvescens*), Atlantic sturgeon (*A. oxyrinchus*), and shovelnose sturgeon (*Scaphirhynchus platorynchus*)	12 (all disomic)	2–12	H_E = 0.46–0.63	Genetic assessment of population structure in the Great Lakes	Welsh et al. (2008)
	316 (13–66) from 10 locations	Lake sturgeon (*A. fulvescens*)	14 (all disomic)	2–12	H_O = 0.514–0.563; H_E = 0.522–0.664	Genetic assessment of population structure in the Ottawa River	Wozney et al. (2011)
Siberian sturgeon (*A. baerii*)	151 (20–48) from 5 locations	Lake sturgeon (*A. fulvescens*), Atlantic sturgeon (*A. oxyrinchus*), and Adriatic sturgeon (*A. naccarii*)	5 (all tetrasomic)	11–18	n.d.	Natural genetic polymorphism and phylogeography	Barmintseva and Mugue (2017)

calculated in the present study. The magnitude of genetic population differentiation strongly depends on the population status (wild or farmed) as well as the geographical scale of sampling (i.e., a stronger differentiation can be expected if the whole distribution range is covered compared to sampling within a single river, lake, or sea basin). Therefore, a comparison with available population genetic studies on lake sturgeon (McQuown et al. 2003; Welsh et al. 2008; Wozney et al. 2011) and Siberian sturgeon (Barmintseva and Mugue 2017) would be suspect and was not attempted.

Conclusion

Although the sample size of the four Russian sturgeon groups examined in the present study was relatively small, the 12 tetrasomic microsatellite loci showed a level of polymorphism that allowed to differentiate between groups and to detect unwanted reductions in genetic variability within hatchery juveniles. The genotypic data obtained with this set of microsatellites can therefore be used to improve the genetic management of captive broodstocks reared for aquaculture purposes (caviar and meat production) or restoration programs (production of stocking material). In particular, these microsatellites can be applied to identify those combinations of spawners that transmit the highest possible genetic variability to the next generation. The process of selecting such suitable spawners can be aided by the software Genassemblage (Kaczmarczyk 2015).

Moreover, the neighbor-joining tree indicates that both the German and Turkish hatchery Russian sturgeons may originate most probably from the Caspian Sea basin, while the Romanian ones are from the Black Sea/Danube River population. Because of its implications for Russian sturgeon supportive stocking programs in the Black Sea basin, this assumption urgently requires more detailed investigations, in particular by including Russian sturgeon samples from the Caspian Sea basin.

Acknowledgements We thank Mr. Peter Groß, fish farm “Rhönforelle”, Gersfeld, Germany for generously providing access to the offspring of the ex situ stocks for sampling. Marian Paraschiv, Ştefan Honţ and Marian Iani of the DDNI Sturgeon Research Group collected most of the tissue samples from the Danube River in Romania.

Funding information This project was sponsored by the COFASP (Cooperation in Fisheries, Aquaculture and Seafood Processing) ERA-NET partners, which has received funding from the European Union’s Seventh Framework Programme for Research, Technological Development and Demonstration under grant agreement no. 321553. Financial support was provided by the German Federal Ministry of Food and Agriculture (BMEL) through the Federal Office for Agriculture and Food (BLE), grant number 2814ERA01G; by the Republic of Turkey, Ministry of Food, Agriculture and Livestock, project number TAGEM/HAYSUD/2015/A11/P01/5; and by the Romanian Executive Agency for Higher Education, Research, Development and Innovation Funding (UEFISCDI), contract number 26/2015.

Compliance with ethical standards All sturgeon samples were collected in conformity with national legislations.**Conflict of interest** The authors declare that they have no conflict of interest.

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Genetics of Aquatic Organisms 2: 35-41 (2018)
www.genaqua.org
ISSN 2459-1831
DOI: 10.4194/2459-1831-v2_2_02

PROOF

RESEARCH PAPER

Development and assessment of a multiplex PCR assay for genetic analyses of microsatellite loci in beluga sturgeon, *Huso huso*

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Received 31 October 2018
Accepted 10 December 2018

Abstract

The survival of the critically endangered beluga sturgeon, *Huso huso*, depends – among others – on an effective fisheries management and supportive stocking. To gather the necessary knowledge on the genetic diversity and structure of still existing wild populations, microsatellite loci from related sturgeon species were tested for their cross-species amplification ability and species-specific loci were newly isolated using 454 GS-FLX Titanium pyrosequencing. Twelve suitable polymorphic microsatellites could be identified and were characterized in 58 beluga sturgeons (18 farmed; 40 wild). The observed total number of alleles per locus ranged from 4 to 18 with moderate to high levels of polymorphism. The wild individuals were significantly more diverse with a mean number of 8.08 alleles per locus than the farmed ones with 4.33 alleles per locus. In contrast, observed heterozygosities did not differ significantly among both groups. Genic and genotypic differentiation across all loci was highly significant between farmed and wild beluga sturgeons. The high sensitivity and discriminatory power of the 12 loci were proven by a very low overall probability of identity for siblings ($PI_{sib}=3.099 \times 10^{-5}$) and a high accuracy of self-classification (98.3%). Thus, this multiplex PCR assay represents a valuable genetic toolbox for species conservation measures.

Keywords: Acipenseridae, conservation genetics, genetic diversity, nuclear DNA, population structure.

Introduction

The beluga sturgeon, *Huso huso* (Linnaeus, 1758), is an anadromous species of the family Acipenseridae historically known from the Caspian, Black, Azov and Adriatic Sea basins. Males reproduce for the first time at 10-15 years and females at 15-18 years. Spawning takes place every 3-4 years from April till June. The complicated pattern of spawning migrations includes one peak in late winter and spring and another one in late summer and autumn (Gesner, Chebanov, & Freyhof, 2010). Individuals migrating in spring spawn in the same year, whereas those migrating in autumn overwinter in the rivers until the following spring. Overfishing at sea and poaching in estuaries and rivers for caviar and meat, spawning habitat losses due to dam construction, inland navigation, and flood protection measures as well as decreased water quality are major threats to the species which resulted in a dramatic decline of its populations (Gesner *et al.*, 2010). The current native wild distribution is restricted to the Black Sea (in the Danube River only) and the Caspian Sea (in the Ural River only). Consequently, the IUCN Red List of Threatened Species classified the beluga sturgeon as "critically endangered" (Gesner *et al.*, 2010). In the immediate future, the survival of the species depends on an effective fisheries management, supportive stocking, combating illegal fishing in several river systems as well as maintaining the remaining spawning and early life stage habitats.

An effective fisheries management as well as the establishment of broodstocks and subsequently of appropriate breeding schemes for supportive stocking programs requires knowledge on the genetic diversity and structure of the remaining wild populations of beluga sturgeon. Microsatellite loci represent a popular and efficient nuclear DNA marker type for genetic investigations in fishes (Liu & Cordes, 2004), and already demonstrated in other sturgeon species their usefulness for the assessment of population structures (Welsh, Hill, Quinlan, Robinson, & May, 2008; Wozney, Haxton, Kjartanson, & Wilson, 2011) and broodstock management (Henderson-Arzapalo & King, 2002; Boscari, Pujolar, Dupanloup, Corradin, & Congiu, 2014). In the present study we describe the

development of a multiplex PCR assay consisting of 12 polymorphic microsatellite loci and assess the variability of these loci in 18 farmed and 40 wild-caught beluga sturgeons.

Materials & Methods

A search in NCBI GenBank (www.ncbi.nlm.nih.gov/genbank/; last accessed June 12, 2018) revealed 227 DNA sequence records for the pure species *Huso huso* or its interspecific hybrids and back crosses with sterlet sturgeon, *Acipenser ruthenus* (Linnaeus, 1758). However, none of these records comprised microsatellite loci. Therefore, two approaches for the development of such markers were taken: (1) tests of 41 microsatellite loci isolated from related sturgeon species for their cross-species amplification ability in beluga sturgeon, and (2) *de novo* isolation of microsatellite loci from a beluga sturgeon genomic library by a commercial service (GenoScreen, Lille, France, www.genoscreen.fr).

To prepare the genomic library fin clips were collected from 18 juvenile beluga sturgeons at a German fish farm and the total genomic DNA was isolated using the DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany) according to manufacturer´s protocols. Ten DNA isolates were pooled and 1.0 µg of the pooled DNA was used for the isolation of microsatellite loci through 454 GS-FLX Titanium pyrosequencing of enriched DNA libraries as described in Malausa *et al.* (2011). The bioinformatics program QDD (Meglécz *et al.*, 2010) was used to analyze the sequences. QDD performs all bioinformatics steps for treating raw sequences: removing adapters/vectors, detecting microsatellites, detecting redundancy/possible mobile element association, selecting sequences with target microsatellites, and designing primers. Among 2898 sequences containing a microsatellite motif, 88 bioinformatically validated primer pairs could be designed. Based on our experience with isolation of microsatellite loci in tench, *Tinca tinca* (Linnaeus, 1758), (Kohlmann & Kersten, 2006), pike-perch, *Sander lucioperca* (Linnaeus, 1758), (Kohlmann & Kersten, 2008), stellate sturgeon, *Acipenser stellatus* Pallas 1771, (Kohlmann *et al.*, 2017a), sterlet sturgeon (Kohlmann *et al.*, 2017b) and Russian sturgeon, *Acipenser gueldenstaedtii* Brandt & Ratzeburg, 1833 (Kohlmann *et al.*, 2018) all sequences with validated primer pairs were ranked according to motif type (tetra- > tri- > di-nucleotide repeats), number of repeats (the higher the better), and PCR product size (>100 bp) considering only sequences with perfect repeats. From this list the 60 top ranking primer pairs were selected for the identification of suitable microsatellites showing consistent amplification, disomic pattern, ease to score, and sufficient variability.

Although the selection procedure of suitable loci was based on separate genotyping of single loci, protocols for microsatellite amplification were already developed for future use of PCR multiplex kits (QIAGEN, Hilden, Germany) and a peqSTAR 96X Universal Gradient thermocycler (Peqlab, Erlangen, Germany). After suitable loci had been identified the software MultiPLX, version 2.1 (Kaplinski, Andreson, Puurand, & Remm, 2005) was used to analyze PCR primer compatibility and automatically find the optimal multiplexing (grouping) solution. Four different dye labels (Atto 680, BMN-6, Cyanine 5 and DY-751; Biomers.net, Ulm, Germany) were assigned to the forward primers. Genotyping of the microsatellite loci was performed on an eight-capillary sequencer CEQ 8000 (Beckman Coulter, Brea, USA) using the Fragment Analysis module of the GenomeLab™ GeXP Genetic Analysis System, version 10.2 (Beckman Coulter, Brea, USA).

The microsatellite variability was examined in the 18 farmed individuals at first but later on extended to samples from 40 wild beluga sturgeons collected in the lower Danube River, Romania during 2004 to 2016. The wild spawners were captured according to the Appendix 1 of the joint Ministerial Order 330/262/2006 (republished by Ord. 82/2012). Shortly after spawning, all individuals were released back into the Danube River. All young-of-the-year (YOY) and juvenile beluga sturgeons captured alive were released after sampling and biometric survey. Tissue samples for genetic analyses were collected from live individuals by minimal invasive sampling (small fragments of the anal fin). No animals were killed during the sampling procedure.

All microsatellite genotypes were initially examined with the Micro-Checker software, version 2.2.3 (van Oosterhout, Hutchinson, Wills, & Shipley, 2004) for scoring errors due to null alleles, stutter bands, and/or large allele dropout. Then, general parameters of microsatellite loci variability (number of alleles, observed and expected heterozygosities) were calculated using GENEPOP 4.0 (Rousset, 2008). Tests for significance of deviations from Hardy-Weinberg equilibrium at single loci and of genic as well as genotypic differentiation between farmed and wild beluga sturgeons were also performed with GENEPOP 4.0. The polymorphic information content (PIC) of loci was determined using Cervus 3.0 (Marshall, Slate, Kruuk, & Pemberton, 1998; Kalinowski, Taper, & Marshall, 2007), and their discriminatory power (probability of identity=PI) was estimated with GENECAP, version 1.4 (Wilberg & Dreher, 2004) applying the more conservative measure of PI for siblings PIsib (Waits, Luikart, & Taberlet, 2001). Finally, the sensitivity of the microsatellite loci was examined by self-classification of the 58 beluga sturgeons applying the Bayesian method and “Leave One Out” procedure of the GeneClass software, version 1.0.02 (Cornuet, Piry, Luikart, Estoup, & Solignac, 1999).

Results & Discussion

In addition to the 41 microsatellites from related sturgeon species, 60 *de novo* isolated beluga sturgeon microsatellite loci were chosen for testing. Among these 101 PCR primer pairs, 12 amplified loci that matched our selection criteria, in particular consistent amplification, disomic pattern and sufficient variability. Four loci (*Aox45*, *Aox*D32, *Aox*D54 and *Aox*D64) originated from Atlantic sturgeon (*Acipenser oxyrinchus* Mitchill, 1815), two loci (*LS-19* and *LS-54*) from lake sturgeon (*Acipenser fulvescens* Rafinesque, 1817), two loci (*Aru13* and *Aru26*) from sterlet sturgeon, one locus (*Anac_c159*) from Adriatic sturgeon (*Acipenser naccarii* Bonaparte, 1836), one locus (*Ag09*) from Russian sturgeon, one locus (*Spl-163*) from shovelnose sturgeon (*Scaphirhynchus platorynchus* (Rafinesque, 1820)), and only one locus (*Hus15*) from the beluga sturgeon genomic library of the present study. These 12 loci were combined into five multiplex PCR sets based on primer compatibility (Table 1).

Optimized PCR reaction mixes consisted of 5.0 µl of master mix and 1.5 µl Q-solution (QIAGEN, Hilden, Germany), 1.0 µl DNA isolate, primers with concentrations as stated in Table 1, and PCR-grade water up to a final volume of 10.0 µl. The PCR program based on QIAGEN recommendations included an initial denaturation at 95 °C for 15 min followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C (multiplex sets 1, 2 and 3), or 63 °C (multiplex sets 4 and 5) for 90 s, and extension at 72 °C for 60 s. A final extension at 60 °C lasted for 30 min.

Tests with the Micro-Checker software did not reveal any evidence for scoring errors due to stutter bands or large allele dropout. Moreover, no indications for null alleles were found at any of the 12 loci of the 18 farmed individuals. In contrast, homozygote excess was observed at three loci (*LS-54*, *Aru13* and *Hus15*) of the 40 wild beluga sturgeons possibly due to null alleles being present at these loci. The observed total number of alleles per locus ranged from 4 to 18 (Table 1) with an average of 8.42. PIC values indicated high levels of polymorphism (PIC>0.500) at ten loci and moderate levels (PICs of 0.272 and 0.288) at the remaining two loci (Table 1). The farmed beluga sturgeons possessed 2 to 6 alleles per locus with a mean number of 4.33; the wild individuals were more variable with 4 to 17 alleles per locus and a mean number of 8.08 (Table 2). Observed heterozygosities ranged from 0.167 at locus *Ag09* to 1.000 at loci *Spl-163* and *Aox*D64 in the farmed and from 0.175 at locus *Hus15* to 0.947 at locus *Aox45* in the wild individuals (Table 2). Differences between the two beluga sturgeon groups were highly significant for mean number of alleles per locus (P=0.003; two-sided *t* test), but non-significant for mean observed heterozygosity (P=0.260; two-sided *t* test). Significant (P<0.05) to highly significant (P<0.01) deviations from Hardy-Weinberg equilibrium were found at four loci in the farmed as well as four loci in the wild beluga sturgeons (Table 2). However, only one locus (*LS-54*) deviated in both groups. The observed deviations from Hardy-Weinberg equilibrium might be caused by the relatively small sample size in case of the farmed beluga sturgeons (n=18) or the possible presence of null alleles and an unequal representation of sampling years in case of the wild beluga sturgeons (the number of individuals per year ranged from 1 to 9).

Genic as well as genotypic differentiation across all loci was highly significant between farmed and wild beluga sturgeons. The probability of identity for siblings (PIsib) at single microsatellite loci ranged from 0.320 at locus *LS-54* to 0.737 at locus *Hus15* (Table 2), and the resulting overall PIsib value was very low: 3.099×10^{-5}. A high sensitivity of the 12 microsatellite loci was also demonstrated by self-classification: 57 out of the 58 individuals (=98.3%) were correctly identified; only one wild beluga sturgeon was assigned into the farmed group.

Conclusion

The described five multiplex PCR sets consisting of 12 microsatellite loci provide sufficient variability and discriminatory power in beluga sturgeon. They represent a valuable toolbox which can be used to characterize the genetic diversity and structure of the remaining wild populations, to aid the development of conservation measures, to establish broodstocks and appropriate breeding schemes for supportive stocking programs as well as to monitor genetic changes in farmed strains used for caviar and meat production.

Acknowledgements

This project was sponsored by the COFASP ERA-NET partners, which has received funding from the European Union's Seventh Framework Programme for research, technological development and demonstration under grant agreement no. 321553. Financial support was provided by the German Federal Ministry of Food and Agriculture (BMEL) through the Federal Office for Agriculture and Food (BLE), grant number 2814ERA01G; and the Romanian Executive Agency for Higher Education, Research, Development and Innovation Funding (UEFISCDI), contract number 26/2015. We thank Mr. Peter Groß, fish farm "Rhönforelle", Gersfeld, Germany for generously providing access to the farmed beluga stock for sampling. Marian Paraschiv, Ștefan Honț and Marian Iani of the DDNI Sturgeon Research Group collected most of the tissue samples from the Danube River in Romania.

Table 1. Characteristics of 12 polymorphic microsatellite loci tested in a total number of 58 *Huso huso* individuals.

PCR multiplex set (annealing temperature)	Locus name (GenBank acc. no.)	Forward primer dye label	Primer concentration (µM)	Primer sequence (5′- 3′)	Repeat motif	Total number of alleles	Allele size range	PIC	Reference
1 (57 °C)	*LS-54* (U72735)	DY-751	0.200	F: CTCTAGTCTTTGTTGATTACAG R: CAAAGGACTTGAAACTAGG	(GATA) (GACA)	18	216-284	0.860	May et al. (1997)
	*Aox*D32 (AY093634)	Cyanine 5	0.070	F: CAGATTTAAGTAAGATAAGCATCAGC R: AAAGCAGCTTGACATAACGG	(TAGA) (CAGA)	8	142-152	0.756	Henderson-Arzapalo and King (2002)
	Ag09 (HG931705)	Cyanine 5	0.050	F: CAGTCGATTTCATTCAAAGCA R: CCTGCCCCTAAACTGATTGA	TATG	5	269-285	0.288	Rajkov et al. (2014)
2 (57 °C)	*Aox45* (AF067813)	BMN-6	0.180	F: TTGTCCAATAGTTTCCAACGC R: TGTGCTCCTGCTTTTACTGTC	AAT	12	115-148	0.849	King et al. (2001)
	Spl-163 (AF276205)	Cyanine 5	0.018	F: TGCTTGTAAACTGCCCCACT R: CCACATGCAGTTTGAGCTGC	GATA	10	168-216	0.785	McQuown et al. (2000)
	*Aox*D54 (AY093636)	DY-751	0.160	F: GAGAACAACGTTTTACTGCAAAC R: GATCATAACTAAAGCTGGCAGG	TAGA	7	170-198	0.564	Henderson-Arzapalo and King (2002)
3 (57 °C)	*LS-19* (U72730)	Atto 680	0.140	F: CATCTTAGCCGTCTGTGGTAC R: CAGGTCCCTAATACAATGGC	TTG	10	127-172	0.836	May et al. (1997)
	*Aox*D64 (AY093637)	Cyanine 5	0.033	F: TTTGTGTAGGGAAATACCCTTG R: TGAGTGCAGCCCTACTGCTC	TAGA	9	216-248	0.811	Henderson-Arzapalo and King (2002)
4 (63 °C)	*Aru26* (MF100775)	DY-751	0.200	F: AAAGCAACAACTCCACCAGG R: TGCCTTGTCTACTGTCCGAA	CT	5	165-173	0.583	Kohlmann et al. (2017b)
	Anac_c159 (KR297008)	Cyanine 5	0.058	F: ACGACATTACTCGAAACTGCTG R: TGATATGCCTTGGAACGCTG	TAT	8	311-341	0.762	Boscari et al. (2015)
5 (63 °C)	*Aru13* (MF100772)	DY-751	0.160	F: TCCACTTTATTCCGTTGTGG R: AGACCGGAATCAAACCCAG	GTT	5	105-123	0.659	Kohlmann et al. (2017b)
	Hus15 (MH605082)	Cyanine 5	0.033	F: AGGGACACGCTCACTGTTTT R: AACAGATGTACAGTCAGACCCG	GT	4	129-137	0.272	this study

Table 2. Variability of 12 polymorphic microsatellite loci in two test panels of 18 farmed and 40 wild-caught *Huso huso* (N_A=number of alleles; H_O=observed heterozygosity; H_E=expected heterozygosity; P_{HW}=exact *P*-value of the Hardy-Weinberg probability test: * P<0.05, ** P<0.01, n.s. non-significant).

Locus	Parameter	Farmed *H. huso*	Wild *H. huso*	Probability of identity for siblings (PIsib)
LS-54	N_A	6	17	0.320
	H_O	0.722	0.667	
	H_E	0.733	0.890	
	P_{HW}	*	**	
*Aox*D32	N_A	5	8	0.378
	H_O	0.944	0.700	
	H_E	0.806	0.750	
	P_{HW}	n.s.	n.s.	
Ag09	N_A	2	5	0.723
	H_O	0.167	0.282	
	H_E	0.157	0.359	
	P_{HW}	n.s.	*	
Aox45	N_A	6	11	0.326
	H_O	0.889	0.947	
	H_E	0.756	0.860	
	P_{HW}	n.s.	n.s.	
Spl-163	N_A	5	9	0.361
	H_O	1.000	0.816	
	H_E	0.798	0.819	
	P_{HW}	n.s.	n.s.	
*Aox*D54	N_A	2	7	0.488
	H_O	0.667	0.725	
	H_E	0.508	0.634	
	P_{HW}	n.s.	n.s.	
LS-19	N_A	6	10	0.334
	H_O	0.833	0.838	
	H_E	0.802	0.846	
	P_{HW}	*	n.s.	
*Aox*D64	N_A	6	8	0.345
	H_O	1.000	0.865	
	H_E	0.756	0.824	
	P_{HW}	*	n.s.	
Aru26	N_A	3	5	0.474
	H_O	0.778	0.605	
	H_E	0.565	0.560	
	P_{HW}	n.s.	n.s.	
Anac_c159	N_A	5	8	0.376
	H_O	0.722	0.684	
	H_E	0.765	0.776	
	P_{HW}	**	n.s.	
Aru13	N_A	4	5	0.406
	H_O	0.889	0.378	
	H_E	0.751	0.745	
	P_{HW}	n.s.	**	
Hus15	N_A	2	4	0.737
	H_O	0.444	0.175	
	H_E	0.356	0.271	
	P_{HW}	n.s.	*	

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