Development of new microsatellite genetic markers for white sturgeon and continued genetic monitoring of the KTOI broodstock.

Deliverable 2) Development of 8 tetranucleotide microsatellite loci for white sturgeon
Deliverable 3) Development of protocol for amplification, and scoring for each locus
Deliverable 4) Completion of written report describing the above as well as the genetic variability of loci and accuracy of new loci for assessing parentage in KTOI broodstock

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Executive Summary

This report details 1) the development of a white sturgeon genomic DNA library to identify additional microsatellite loci and 2) the use of eight new microsatellites for parentage assignment in the Kootenai River conservation aquaculture program. Kootenai River broodstock (2001, 2003, 2004) and larvae from eight full-sib families (produced in 2004) were genotyped at eight new microsatellite loci, and these data were combined with the existing microsatellite dataset collected by Rodzen et al (2004a). The full microsatellite dataset was used to assign parentage to the eight full-sib families for two scenarios: 1) when only parents were included in analysis, and 2) when all broodstock of known sex were included in analysis.

The addition of eight microsatellite loci resulted in a substantial increase in the accuracy of parentage assignment for both scenarios. Greater assignment accuracy allowed for the identification of errors that could not be resolved in previous analyses. The implementation of a confidence threshold (δ), increased the assignment accuracy, but reduced the total number of assignments that could be made for each family. The costs and benefits associated with using the confidence threshold to assign parentage within the Kootenai River conservation aquaculture program are discussed.

The inclusion of additional microsatellite markers might improve success of parentage assignment in the Kootenai River population. However, the reduced level of genetic diversity within the Kootenai River population may continue to impede our ability to substantially further increase power in these analyses. We recommend analyzing the current microsatellite dataset with additional software programs in an attempt to increase assignment accuracy.

Introduction

The Kootenai River white sturgeon (*Acipenser transmontanus*) population inhabits Kootenay Lake and the Kootenai River between Kootenai Falls and Bonnington Falls (Kincaid 1993). It has been naturally isolated from other white sturgeon populations for approximately 10,000 years (Northcote 1973) and may have become locally adapted to its unique environment (Paragamian and Kruse 2001). Wild recruitment in this population has declined precipitously since the 1970's and is currently thought to be nonexistent (Anders et al. 2002). Because of its uniqueness and vulnerability, the Kootenai population was listed as endangered under the Endangered Species Act in 1994 (USFWS 1994).

To mitigate for the lack of natural recruitment, the Kootenai Tribe of Idaho initiated a conservation aquaculture program in 1990 to introduce hatchery produced white sturgeon to the Kootenai River (Ireland et al. 2002). In this program, wild broodstock are captured and spawned in a hatchery facility and four to twelve families of offspring are created annually. The offspring are reared in the hatchery for 15-24 months, at which point they are released into the river (Ireland et al. 2002). The Kootenai Tribe of Idaho has taken an active role in the genetic management of their conservation hatchery program. A major goal of the original genetic management plan for the hatchery program is the preservation of existing genetic variation in the population (Kincaid 1993). Genetic monitoring of the Kootenai River population conducted by the Genomic Variation Lab (GVL) at the University of California, Davis has indicated that broodstock selected by the hatchery program represent 94% of the genetic diversity available within the population (Rodzen et al. 2004a).

Another genetic concern for the Kootenai River hatchery program is inbreeding depression (Kincaid 1993). Inbreeding depression is a decrease in population fitness that results from increased homozygosity (due to mating between close relatives) and the expression of deleterious recessive alleles. As the number of wild adults in the population continues to dwindle, it is necessary to consider that by the next decade, a majority of the adults captured for breeding may originate from the hatchery program. Because only a limited number of families are created in the hatchery program in any given year, it is possible that potential broodstock may be relatives of the first (i.e. full sibs) or second order (i.e. cousins). It becomes necessary to elucidate the relatedness between potential broodstock in order to prevent the mating of close relatives and which may increase the potential for inbreeding depression in the already vulnerable population.

One way to identify familial relationships between potential broodstock is through parentage assignment. In this analysis, highly polymorphic loci such as microsatellites can be used to identify the parents of putative broodstock. Parentage assignment has been used successfully in a number of taxa to better understand mating systems, individual mating success, and social structure within natural populations (Girman et al. 1997; Marshall et al. 1998; Gerber et al 2000; Jones and Ardren 2003; Rudnick et al. 2005). A study by Rodzen et al. (2004b) revealed that parentage analysis with eight polymorphic microsatellite markers could successfully identify the parents of full-sib families of white sturgeon created in an aquaculture program. These techniques were applied by the GVL to assign parentage within the 2004 year class produced by the Kootenai River hatchery program. However, low genetic variability within the Kootenai population lead to low assignment success relative to the levels of success observed in the

more variable aquaculture stock.

The objective of this study was to develop new microsatellite markers that could be used to increase the accuracy of parentage assignment with the Kootenai River hatchery program. We created a genomic DNA library for both white and green sturgeon (*A. medirostris*) to identify additional polymorphic microsatellite loci that amplified consistently in white sturgeon. We genotyped broodstock (2001, 2003, 2004) and eight full-sib families (2004) at the new loci and merged this dataset with the existing genotype data that had been collected for these individuals by the GVL in 2004. We then used the merged microsatellite dataset to conduct parentage analysis with 1) only known parents for the 2004 progeny and 2) all broodstock of known sex captured in 2004. Assignment accuracy with the merged sixteen marker dataset was compared to that achieved with the original eight marker dataset.

Materials and Methods

Marker Development

We created a combined genomic DNA library for green and white sturgeon to maximize our efficiency in developing new microsatellite markers for two separate projects. DNA from two white sturgeon and green sturgeon individuals was extracted using a phenol/chloroform/isopropanol procedure as described in Sambrook (1989). Genomic DNA from these individuals was pooled for magnetic bead enrichment following the procedure detailed in Jones et al. (2000). DNA was digested with seven restriction enzymes (Rsa I, Hae III, Bsr B1, Pvu II, Stu I, Sca I, and Eco RV) and enriched for repeat motifs (Bork et al. 2005). Enriched DNA was cloned into pUC19

plasmid and transformed into E. coli strain DH5α. Recombinant clones containing repeat motifs were selected for sequencing on an ABI 377 genetic analyzer. All sequencing was conducted by Genetic Identification Services (GIS; Bork et al. 2005).

A total of eight microsatellite libraries were screened (AAAC, AAAG, AAAT, TACA, CATC, TAGA, CAGA, and GTCA) and 191 microsatellites were identified using the program mreps (http://bioinfo.lifl.fr/mreps/). Microsatellites containing fewer than 3 repeat units were excluded from further consideration. Remaining sequences were checked for duplication and a total of 161 unique microsatellite loci were available to screen for polymorphism (Bork et al. 2005).

Unlabeled primers were designed from microsatellite flanking regions using the program Primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Polymorphism screens were conducted for both green and white sturgeon using four individuals from each species. PCR conditions for microsatellite amplification were as described in Israel et al. (2004). The thermal profile for initial amplification consisted of initial denaturation at 95°C for 90 s, followed by 35 cycles of 95°C for 1 min, 56°C for 45 s, and 72°C for 2 min, with a final extension of 72°C for 5 min. Formamide loading dye was added to each reaction and amplified DNA was separated by size on 5% denaturing polyacrylamide gel run at 65W. A 400 bp ladder (The Gel Company) was included in one lane on each gel for approximate fragment sizing. Bands were stained with a Sybr-Green™ (Invitrogen) – agarose overlay as described by Rodzen et al. (1998) and visualized with a FluorImage 595 (Molecular Dynamics).

Microsatellites exhibiting polymorphism across four white sturgeon individuals were identified and subjected to further screening. A screening set of sixteen white

sturgeon from several populations was assembled and used to examine the extent of polymorphism in 29 loci. PCR amplification and polyacrylamide electrophoresis were conducted as described for our initial microsatellite screens of four white and green sturgeon individuals.

A new set of eight additional microsatellite loci was added to the existing suite of markers described by Rodzen et al. (2004b) for the parentage analysis based on observed polymorphism and consistency of amplification. Included in this subset was a locus developed for the Chinese sturgeon, *Acipenser sinensis* (Zhu et al. 2005), which was found to cross-amplify well in white sturgeon. These eight loci were optimized to develop locus specific amplification parameters (Table 1).

Samples

A total of 255 Kootenai River white sturgeon samples were genotyped at eight microsatellite loci. Sixty-three of these samples had been collected from broodstock used in the Kootenai Tribe of Idaho (KTOI) conservation hatchery program in 2001 (N=16), 2003 (N=17), and 2004 (N=30). The remaining 192 larval samples originated from eight full-sib families (24 offspring/family) produced by crossing 8 sires and 5 dams in the KTOI hatchery in 2004.

Genotyping

DNA was extracted from 63 broodstock sturgeon using the PureGene™ DNA Purification System Tissue Kit (GentraSystems), while progeny DNA extraction was conducted following the protocol in Rodzen and May (2002). PCR was performed for

each individual in PTC 220 Dyad thermocyclers (Bio-Rad) with fluorescently labeled primers for the eight newly developed microsatellite loci. PCRs contained 1.0 ul of DNA extract (~5 ng), 0.8 ul dNTPs (2.5 mM ea), 1.5 – 3.0 mM $MgCl_2$, 1.0 of each primer (5 uM), 0.4 U of taq polymerase (Promega) in 1X reaction buffer (10 mM Tris-HCl, 50 mM KCl, and 0.1% Triton® X-100), diluted to a total volume of 10 ul with dH_20. Locus-specific PCR conditions, including thermal profiles, are detailed in Tables 1 and 2.

PCR products were separated by size in a 5% denaturing polyacrylamide gel run on a Bio-Rad BaseStation DNA Fragment Analyzer. A fluorescently labeled size standard (Rox 400HD; Applied Biosystems) was included in each lane to allow for accurate allele sizing. Genotyping was conducted using the Bio-Rad software program Cartographer vers 1.2.6.

Genotyping in white sturgeon is complicated by the fact that the species' genome is characterized by varying gene copy numbers, ranging from disomy (two allele copies/locus) to octosomy (8 allele copies/locus). An alternative method of genotyping was employed, wherein each microsatellite allele was treated as a dominant locus and scored as either present or absent (Rodzen et al. 2004a, b).

Data Analysis

Genetic diversity of the Kootenai broodstock (2001, 2003, 2004) and the 2004 progeny at the new microsatellite loci was estimated by calculating the number of alleles per locus. Genotypes from the new eight microsatellite loci were combined with the original genotypic data collected for these individuals by Rodzen et al. (2004b), yielding a dataset that included sixteen polymorphic microsatellite loci. The log-likelihood (LOD)

method of Gerber et al. (2000) was used to assign parentage to offspring of the eight full-sibling crosses. To calculate LOD scores, we took the log of the likelihood that individual X is the parent of offspring A and divided it by the likelihood that individual X and offspring A are unrelated (Gerber et al. 2000). LOD scores were then summed across all loci. To evaluate the significance of such assignments, the statistic delta (δ) was employed to evaluate how much more likely the "best" parent was to be the true parent than was than the "second best" parent identified (Marshall et al. 1998). Delta is simply the difference of the LOD scores between the most likely and second most likely parent of a given offspring. For example, a δ of 3 for a certain possible parent means that particular animal was 10^3 (=1000) times more likely to be the true parent than the second most likely possible parent. A distribution of δ values for correct and incorrect assignments was plotted (Figure 1) and the point where the two distributions intersected was selected as the critical δ, as described in Rodzen et al. (2004a). The two distributions intersected at a δ of 2.5. Thus, if the most likely animal is $10^{2.5}$ (=316) times more likely to be the true parent over any other animal, it is very likely that this animal is in fact the true parent and not a "false positive." These data analysis techniques were identical to the methods used for parentage analysis in the Rodzen et al. (2004a) report to the KTOI.

The sixteen microsatellite loci were tested in their ability to assign parentage in two scenarios: when only known parents (N=13) were included in analysis and when all broodstock of known sex (N=19) were included in analysis. In situations where the results of parentage analysis ran contrary to expectations (e.g. Family KT193C; see Results) the dam or sire assigned as the most likely parent of a majority of offspring in the family was identified as the true parent. The percentage of correct assignments to

both dam and sire in each scenario were calculated with and without employing the δ statistic in order to quantify increases in accuracy when employing the more stringent assignment criteria.

Results

The number of alleles per locus at the new microsatellite loci ranged from 3 – 9 (Table 1). Diversity at these loci was slightly higher than that observed at the eight loci developed by Rodzen et al (2004b). When datasets were combined, a total of 88 alleles were available for parentage analysis from the 16 available loci.

The addition of the eight microsatellite loci to the existing genotype dataset for the Kootenai River notably increased our ability to assign parentage of known full-sib families. We will first consider assignments made without utilizing the δ criterion. In scenario 1, where only known parents were analyzed, assignment of offspring to the correct sire or dam ranged from 29-100% and 50-100%, respectively (Table 3). Mean assignment accuracy across families was 81% in sires and 85.5% in dams. When family KT270D was not considered, assignment accuracy in sires increased to 88% (see below). This represents a substantial improvement in assignment success for both sires and dams with the addition of the new markers.

As in Rodzen et al. (2004a), assignment accuracy in scenario 2, where all parents and non-spawned broodstock of known sex were included in analysis, was lower than that in scenario 1 (sires: 8-100%, dams: 50-96%; Table 3). Assignment accuracy to correct dams remained similar between scenarios 1 and 2; however, a decrease in assignment success was observed for sires in scenario 2. Nonetheless, mean assignment

accuracy in scenario 2 increased by 20-30% with the addition of the eight microsatellite markers.

In both scenarios 1 and 2, a majority of offspring (87.5% and 83%) from family KT193C were assigned to dam 7F7D100A59, while hatchery records suggest that dam 7F7E6A4306 is the maternal parent. A number of genetic discrepancies were noted in this family during data collection, such as a majority of offspring possessing alleles not observed in either parent. It was suspected that either an error in data transcription or gamete contamination occurred at some point during the spawning process. No offspring were assigned to dam 7F7E6A4306 in either scenario, while a majority was assigned to 7F7D100A59. When the δ criterion was employed, all offspring were assigned to 7F7D100A59 (see below; Table 4). Therefore, dam 7F7D100A59 was most likely the true maternal parent of family 193C.

Another possible error was identified in family KT270D. When additional loci were included in parentage analysis, assignment success in this family actually was lower than success observed with fewer loci (Table 3; Table 4). We believe this case may represent a misidentification of progeny samples or contamination during the spawning process. The maternal parent of family KT270D, 7F7D113372, also was spawned with a male (7F7F3RB5E5C) from which no tissue sample was collected. It is possible that the our progeny samples for the family represent half-sibs from both sires 7F7D11270D and 7F7F3RB5E5C, or progeny from only the 7F7D113372 x 7F7F3RB5E5C cross. A tissue sample from 7F7F3RB5E5C would be required to distinguish between these possibilities.

When assignment results were interpreted using the critical δ of 2.5 as a confidence threshold for parentage analysis, the number of possible assignments

decreased to 72% and 74% for sires and dams in scenario 1 and to 56% and 74% in scenario 2 (Table 4). However, assignment *accuracy* increased greatly when the δ 2.5 criterion was used. In scenario 1, the correct sire and correct dam were selected 100% of the time in five of eight families (Table 4). In scenario 2, the correct dam was selected as the true maternal parent 100% of the time in five families, while no families had 100% assignment accuracy in this scenario when δ was not employed (Tables 3, 4). However, the number of offspring that could be assigned to both a dam and sire using the δ criterion was low, ranging from 12.5 – 75% in scenario 1 and 17-75% in scenario 2 (Table 5).

Discussion

The inclusion of eight additional microsatellite markers did increase our ability to identify correct parents of known full sib families through log-likelihood methods. The increased assignment accuracy has allowed us to identify mistakes that could not be resolved in previous analyses with fewer microsatellites. For example, the true maternal parent in family KT193C was identified and the supposed paternity in family KT270D was placed in question. Assignment success with eight and sixteen microsatellite loci is still highest in the Kootenai River population when only fish that were actually used to produce families are included in parentage analysis. Because the population is small and recruitment has been limited, it is possible that high levels of relatedness exist among adults in the Kootenai population when compared to other more genetically variable populations (e.g. lower Columbia River).

By increasing the number of adults included in parentage analysis for scenario 2, we also increase the probability that the potential parent pool contains individuals that are

first order relatives (full sibs or parent-offspring). The presence of closely related possible parents in the broodstock may confound parentage analysis (Marshall et al. 1998) because parentage assignment is most accurate when genetic differences between individuals are large. For example, consider family KT2972. In family KT2972, the number of assignments to the correct sire, 7F7D0E2972, was high in scenario 1 (96% and 100% with and without δ, respectively). In scenario 2, 38% of the offspring were assigned to sire 7F7D0F7744 when the δ criterion was not employed. When δ was used as a threshold, this number decreased to 17%. It is possible that sire 7F7D0F7744 is the father or brother of the true sire 7F7D0E2972. However, 7F7D0F7744 also was incorrectly identified as the sire of 66% of the offspring in family KT6532. It is also possible that 7F7D0F7744 possesses a number of high frequency alleles that may be shared by the progeny due to chance.

Using the δ 2.5 criterion as a measure of assignment confidence increased the number of correct assignments in both scenarios 1 and 2. However, the number of assignments that could be made for each offspring declined when a critical δ of 2.5 was employed. This suggests that the costs and benefits of using the δ criterion when assigning parentage in the Kootenai River white sturgeon hatchery program must be considered.

If δ 2.5 is used, relationships between fewer potential broodstock may be unambiguously characterized, further reducing the number of fish used for spawning in a given year. However, if δ 2.5 is not utilized, the risk of making an assignment error is greater and crosses between siblings or half-siblings mistakenly may be made. The decision to use or not to use the δ criterion may depend on the health of the fishery when

hatchery progeny are being sampled for use as broodstock (which may occur within this decade). If managers have a large number of potential broodstock from which to select in a given year, using the δ 2.5 criterion to determine the parents of these potential broodstock animals may be advisable. If the δ 2.5 criterion is used, managers could identify sib groups within the pool of broodstock animals with a high level of confidence, and thus the accidental crossing of full sibs could be avoided. However, if the number of available sexually mature adults decreases over time and the number of potential broodstock is small, it may be acceptable to maximize the number of matings possible. In this case, it may be more advisable to base decisions on the best available parentage data without utilizing the critical threshold. Of course, parentage analysis of collected broodstock animals is only possible if DNA or tissue samples were taken from their parents when they were originally produced in the hatchery.

In the future, we would advise using Kincaid's suggested number of breeders per year as the bare minimum number of fish to spawn and cross. This does not differ from the recommendations of the Rodzen et al. (2004a) report to the KTOI. If additional broodstock of hatchery origin can be collected, then the δ 2.5 criterion can be used in parentage assignment. Individuals whose parents cannot be assigned with high probability (i.e. low δ score) can be omitted from spawning. Given the results of this phase of the study, this may involve collecting and screening up to three times as many fish as are actually needed for spawning to attain the minimum numbers recommended by Kincaid (1993).

The inclusion of more microsatellite markers might improve success of parentage assignment in the Kootenai River population. Sixteen microsatellites yielded 88

dominant loci for analysis, while 100-200 dominant loci are recommended for optimal parentage resolution with the log-likelihood method of Gerber et al. (2000). An additional technique to resolve relationships within the Kootenai River population might be progeny array construction. Some researchers have successfully used this technique identify full-sib families and estimate a minimum number of spawning green sturgeon in a natural system (Josh Israel, personal communication). However, low levels of resolution between half-siblings and unrelated individuals in this technique may lead to misidentification of half-siblings as non-relatives. Spawning half-siblings is less harmful than spawning first order relatives (e.g. parent-offspring pairs, full siblings) but ultimately may lead to inbreeding depression over time.

In the future, we intend to conduct parentage assignment with additional algorithms and software programs in an attempt to increase our accuracy (Danzmann 1997; Gerber et al. 2003). However, the reduced level of genetic diversity within the Kootenai River population may continue to impede our ability to substantially further increase power in these analyses. Regardless, the inclusion of the eight additional microsatellites developed in the Genomic Variation Laboratory has appreciably increased the accuracy of parentage assignment for full-sib families produced by the Kootenai River white sturgeon conservation aquaculture program.

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Table 1. PCR conditions for the eight additional microsatellite loci used in this study. Number of alleles in the Kootenai River broodstock and progeny are given (N = 256). Thermal profiles details are provided in Table 2.

Locus	No. Alleles	$MgCl_2$ (mM)	Thermal Profile
As015	5	2.5	Neilstur
Actm 2	4	1.5	Nlstur60
Actm 35	9	3.0	Neilstur
Actm 43	9	2.0	Neilstur
Actm 52	8	2.0	Promegataq
Actm 53	3	2.0	Promegataq
Actm 110	6	2.0	Neilstur
Actm 177	3	2.5	Neilstur

Table 2. Cycling parameters used for PCR in this study.

Promegataq	Neilstur	Nlstur60
1: 95°C for 90 s	1: 95°C for 2 min	1: 95°C for 2 min
2: 95°C for 1 min	2: 95°C for 30 s	2: 95°C for 30 s
3: 56°C for 45 s	3: 56°C for 30 s	3: 60°C for 30 s
4: 72°C for 2 min	4: 72°C for 90 s	4: 72°C for 90 s
5: Go to 2, 35 x	5: Go to 2, 35 x	5: Go to 2, 35 x
6: 72°C for 5 min	6: 72°C for 5 min	6: 72°C for 5 min
7: 10°C hold ∞	7: 10°C hold ∞	7: 10°C hold ∞

Table 3. Percentages of correct assignment for dams and sires by family without the use of the delta criteria. Previous assignment successes using eight original microsatellite loci are in parentheses. Both scenarios 1 (known parents only) and 2 (all broodstock of known sex) are included in this table.

	Scenario 1		Scenario 2	
Family	% Correct sire assignment	% Correct dam assignment	% Correct sire assignment	%Correct dam assignment
KT261A	92 (38)	96 (42)	87.5 (17)	92 (42)
KT2972	96 (17)	92 (42)	62 (0)	92 (42)
KT270D	29 (42)[a]	83 (50)	8 (33)[a]	83 (50)
KT3672	100 (88)	96 (96)	100 (88)	96 (96)
KT6453	96 (92)	50 (42)	92 (46)	50 (42)
KT627C	92 (100)	87.5 (75)	92 (83)	92 (79)
KT193C	75 (79)	87.5* (92)[b]	75 (63)	83* (92)[b]
KT6532	67 (21)	92 (92)	33 (17)	92 (83)
Mean	81 (60)	85.5 (66)	69 (43)	85 (66)

*Assignment to 7F7D100A59

[a]True sire likely not sampled

[b]Note that a direct comparison is not possible here because offspring were assigned to a different dam in previous analyses

Table 4. Percentages of possible assignments and accurate assignments made using the delta criterion (critical value = 2.5). Both scenarios 1 (known parents only) and 2 (all broodstock of known sex) are included in this table.

	Scenario 1		Scenario 2	
Family	% Correct sire assignment	% Correct dam assignment	% Correct sire assignment	% Correct dam assignment
KT261A	100	100	91	100
KT2972	100	95	83	95
KT270D	40[a]	94	12.5 [a]	94
KT3672	100	100	100	100
KT6453	100	47	100	53
KT627C	100	100	94	100
KT193C	87	100*	67	100*
KT6532	80	100	83	100
Mean	88	92	79	93

*Assignment to 7F7D100A59

[a]True sire likely not sampled

Table 5. Percentages of possible assignments to sires, dams, and both sire and dam for each individual using the delta criterion (critical value = 2.5). Both scenarios 1 (known parents only) and 2 (all broodstock of known sex) are included in this table.

	Scenario 1			Scenario 2		
Family	Sire	Dam	Both	Sire	Dam	Both
KT261A	71	83	58	46	79	37.5
KT2972	87.5	87.5	75	50	87	42
KT270D	21[a]	67	12.5*	33[a]	71	17*
KT3672	96	75	71	96	79	75
KT6453	87.5	71	58	54	62.5	25
KT627C	87.5	75	62.5	71	79	58
KT193C	62.5	67	33	50	67	37.5
KT6532	62.5	71	42	50	71	42
Mean	72	74	51.5	56	74	42

*Due to low assignment success in sire (7/24 and 8/24 assignments possible, respectively)

[a]True sire likely not sampled

Figure 1. Distribution of δ values for incorrect and correct parentage assignments in known full-sib families. Assignments of progeny in Family 193C to dam 7F7D100A59 were considered to be correct assignments. The critical δ value is identified by the first intersection of the incorrect and correct δ value distributions (in box).