

Letter

Standardising multi-laboratory microsatellite data in Pacific salmon: an historical view of the future

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Abstract – For at least 15 years, multiple Pacific Rim Laboratories have cooperated to standardise the collection of Pacific salmon genetic data. For species such as Chinook salmon and chum salmon, allozyme electrophoretic data sets now include hundreds of populations sampled over multiple years throughout the north Pacific. More recently, microsatellite DNA markers have emerged as a new cornerstone of Pacific salmon genetic research. The allozyme experience provides at least two important lessons regarding shared, standardised databases. First, interlaboratory standardisation is sufficiently costly and time consuming that little progress is typically made in the absence of specific fishery management and conservation needs; thus immediate needs will direct future standardisation. Secondly, justified or not, there are significant concerns regarding intellectual propriety and other perceived privileges associated with unpublished genetic data that are shared among laboratories. This article describes challenges to genetic standardisation relative to new research goals, along with specific suggestions for meeting those challenges.

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Introduction

The use of microsatellite markers in Pacific salmon (*Oncorhynchus* spp.) has increased dramatically in recent years as it has throughout ecological genetics and throughout the world. Microsatellites are now used widely in Pacific salmon for descriptive population genetics (Nelson et al. 1998; Olsen et al. 1998; Wenburg et al. 1998; Banks et al. 2000; Ford et al. 2004), historical biogeography (Nielsen & Fountain 1999), fishery management (Scribner et al. 1998; Beacham & Wood 1999; Small et al. 2004), reproductive biology (Bentzen et al. 2001; Ramstad et al. 2003), genomics and mapping (Jackson et al. 1998; Danzmann et al. 1999; Sakamoto et al. 1999) and conservation biology (Banks et al. 1996; Nielsen et al. 1999; Banks et al. 2000; Olsen et al. 2000; Nelson et al. 2003). Small local studies conducted by single research groups have grown to include tens of

thousands of fish from scores of populations across multiple river basins. Microsatellite markers have come to play similarly important roles elsewhere in the world, and although we focus here on Pacific salmon in North America, we know that standardisation is an issue in many species, particularly in a conservation context. Recent conservation genetic literature underscores the breadth of microsatellite use worldwide (Moran 2002).

At the same time, there is increasing frustration among managers and conservation biologists regarding the inability to merge regional data sets generated in different laboratories. In order to make comparisons between data sets, whether microsatellite data or some other class of genetic data, there must be a set of loci or characters that is examined in common between laboratories. A second impediment to fusing data sets is that alleles or character states within a locus, nearly always have different designations in

different laboratories. Microsatellite alleles are typically identified by size in base pairs (in salmon genetics), and while this might seem to simplify coordination among laboratories, the absolute sizing of microsatellite alleles is complicated by cross-platform differences in electrophoretic conditions (see below). Geneticists agree on the need to standardise data, yet little progress has been made.

Many of the issues now faced in the implementation of microsatellite markers at large geographic scales were first confronted by salmon geneticists over 20 years ago in the coastwide standardisation of allozyme data (Shaklee & Phelps 1990; Shaklee et al. 1990, 1999; Utter 1991; Utter & Ryman 1993). These issues were typically resolved through formal collaborations that included exchanges of information on electrophoretic conditions, buffer systems, models used to interpret complex tetraploid banding patterns and photographs of gels. The ultimate verification of genotyping consistency came through the systematic exchange of tissues among laboratories. Significant effort was often required to resolve standardisation issues (Shaklee & White 1991), and progress was slow throughout the 1980s and 1990s, except in cases where management directives provided specific and immediate motivation.

In this article, we discuss practical issues related to current standardisation of microsatellite data for specific research objectives. We then summarise four critical aspects of future microsatellite standardisation: (i) geographic scale of standardisation, (ii) selection of loci, (iii) standardisation of allele designations and (iv) sharing of unpublished data. Our comments are offered to a general audience interested in the issue of combining microsatellite data collected in different laboratories, including population geneticists, ecologists, conservation biologists, fish and wildlife managers and wildlife forensic scientists. A challenge is to provide breadth yet address some of the fairly technical aspects of microsatellite variation.

Standardisation of microsatellite data: practical issues

Precise but inaccurate sizing of microsatellite alleles

One important practical issue confronting microsatellite standardisation relates to electrophoretic mobility and the size estimation of alleles. The fluorescent electrophoretic instruments in current widespread use in salmon genetics employ internal lane standards and are extremely precise (generally <0.3 bp between assays of the same allele). However, size estimates between platforms commonly differ by 3–6 bp or more (Fig. 1; LaHood et al. 2002) because of slight differences in the electrophoretic properties of slab gel systems as opposed to capillary

electrophoretic instruments, for example. Even when laboratories use the same molecular weight size standards, the same sizing algorithm and the same fluorescent labels, standardised sizing can be complicated by the fact that microsatellite alleles and fragments in the standard have different sequences and base composition resulting in differential mobility under different electrophoretic conditions (Gill et al. 1994; Haberl & Tautz 1999). Therefore, size estimates – and hence allele designations – are really only relative to other estimates on the same instrument. Moreover, even the relative allele designations derived from a single instrument can break down because of the mobility characteristics of particular sequences (see below).

Anomalous mobilities

Most salmon microsatellite loci exhibit alleles that differ from one another by multiples of 2 or 4 bp (dinucleotide and tetranucleotide repeats, respectively); however, some pairs of alleles can show small but consistent mobility differences (<0.5 bp) from the general repeat motif, apparently violating the 2 or 4-bp repeat increment. These slight mobility differences presumably relate to base substitutions in the regions flanking the tandem repeat or imperfect repeats within the array. That is, two alleles with the same number of repeat units can differ slightly in mobility because of sequence differences. Although these mobility differences are consistent, they complicate automated genotyping, and the alleles must often be pooled. The choices for pooling of alleles (often referred to as ‘binning’ with microsatellite alleles) must be clearly described in the metadata associated with future microsatellite data sets, if they are to be readily integrated with data collected in other laboratories.

Another implication of base composition and mobility is that it is often impossible to give allele designations that consistently reflect even the relative sizes of the microsatellite alleles. For example, if base composition of the microsatellite alleles produces an increased mobility relative to the same sized fragment in the standard, then the apparent size increment between alleles will be larger than the actual repeat unit, say 2.1 bp rather than the actual 2.0 bp. If the smallest allele is designated by size (e.g., 110.0 bp = ‘110’, 112.1 bp = ‘112’, etc.), then the 20th allele will receive a designation that is a full 2 bp smaller than its estimated size (e.g., 152.0 bp = ‘150’). These numbers are given for simplicity. An actual example is shown in Fig. 1. For a locus with a broad bimodal distribution, these patterns can be subtle, and the likelihood of allele designation errors between platforms is high (Haberl & Tautz 1999; Weeks et al. 2002).

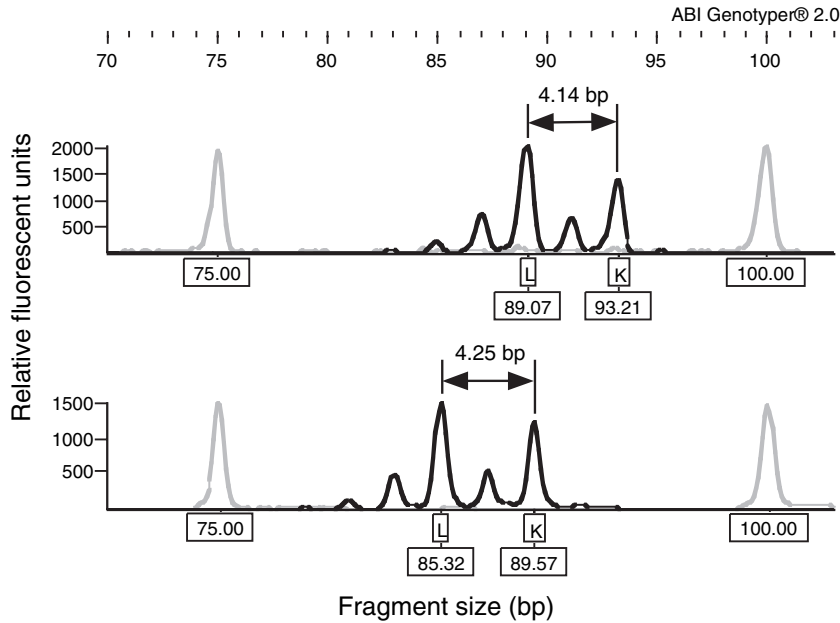


Fig. 1. For the same Chinook salmon individual with microsatellite genotype ‘LK’ (*Ots2* labelled with FAM), electropherograms show substantial differences in the estimated allele sizes produced by two electrophoretic instruments (Applied Biosystems Incorporated (Foster City, California, USA) 377 Genetic Analyzer below and ABI 310 above). In addition, a greater than 2.0-bp estimated repeat unit increment is shown between alleles for this dinucleotide locus (2.07 and 2.13-bp increment on each instrument, respectively). Together these two common attributes of microsatellites complicate cross-platform standardisation as well as consistent naming of alleles according to size within a given platform (Haberl & Tautz 1999). 75 and 100-bp peaks (grey) represent fragments in the same molecular weight size standard. Estimated sizes of each fragment in the test genotype (black) are shown beneath the arbitrarily designated allele names, ‘L’ and ‘K’ (LaHood et al. 2002).

High levels of variability

Although some microsatellite loci have only two alleles and may be fixed in many populations, most microsatellites in current use are highly polymorphic (DeWoody & Avise 2000). Some microsatellite loci have 30 or more alleles in a single population sample of 48 fish. Even with more commonly used microsatellite loci with 8–12 alleles, high allelic diversity naturally has important implications for genetic standardisation, both in the way microsatellites are assayed and in the way the resulting data are analysed. Microsatellites are often sufficiently variable that it can be risky to make inferences about allele identity among regional data. In addition to broad and complex size distributions, some microsatellite loci have complicated imperfect repeat structures. As observed by researchers seeking to standardise allele designations among human genomics laboratories, ‘it would not suffice to attempt to align alleles by typing [or sequencing] only one or two controls in common’ (Weeks et al. 2002).

Current motivation for standardised genotyping

In planning for future genetic standardisation in Pacific salmon, it is important to consider current activities and short-term goals in the context of

broader, long-term objectives. Without clearly defined goals that are driven by specific biological and management objectives, standardisation efforts will be inefficient, and it will be difficult to get broad consensus in the research community for standardisation activities. Although the focus here is Pacific salmon, genetic standardisation is an important issue in many other species often motivated by similar objectives.

Standardisation of genetic markers typically follows a bottom-up approach. That is, allele standardisation happens first within a single laboratory, perhaps between individual researchers, between tissue types or between platforms/methods within a given study. Eventually, as the geographic scope of the study within a laboratory expands, standardisation is required within and between regions and may begin to involve other laboratories. Finally, regional standardisation efforts are combined to produce coastwide baseline data and inevitably include multiple laboratories. Viewing the scope of standardisation as local, regional and species-wide (or at least ‘coastwide’), current standardisation efforts for microsatellite data collection are primarily directed at a regional scale, e.g., current efforts to standardise genetic data among laboratories working on Columbia River steelhead. Already, however, broader, more ambitious efforts are underway (see <http://www.fisheries.org/>

genetics/newsletters/apr2004.shtml#content2 for range-wide microsatellite standardisation in Chinook salmon). Thus, it is worth considering various current goals of genetic standardisation.

Conservation and management units

Conservation and recovery goals have come to dominate Pacific salmon genetics. Many current conservation problems require genetic data to make informed management decisions (National Research Council 1996; McElhany et al. 2000; Moran 2002). In most cases, specific genetic data are not available to assist in these decisions. As a result, it is often the case that geneticists and managers must assemble whatever bits of incompatible genetic data are available. It is not unusual to be confronted with only semi-overlapping sets of loci and incompatible allele designations. There is increased interest in comprehensive genetic data of the kind needed for identification of evolutionarily significant units (ESUs; Waples 1991) and smaller management or conservation units within ESUs. The hope among managers is that the community of individual genetics laboratories will eventually converge on suites of loci and allele designations that will allow the combination of data collected in different laboratories and for different classes of studies.

Mixture analysis: from harvest to habitat use

The initial motivation for standardising allozyme data for Pacific salmon was to analyse mixed-stock fisheries (e.g., Shaklee & Phelps 1990). Because harvest of adult fish often targets stocks originating from numerous regions, mixture analyses using genetic data (Pella & Milner 1987) typically require geographically broad baseline data sets. Following more than two decades of allozyme applications (Grant et al. 1980; Shaklee et al. 1999; Winans et al. 2001), microsatellites have recently been widely employed to analyse adult fish harvests (Beacham & Wood 1999; Beacham et al. 2001; Winans et al. 2004). The shift from allozymes to DNA analysis is occurring for four reasons. First, allozymes are widely considered to require lethal sampling (but see Van Doornik et al. 1999), whereas nonlethal sampling is preferred when conducting baseline surveys of depressed populations. Secondly, tissues taken for DNA analysis can be stored and shipped at ambient temperature, eliminating the need for dry ice or liquid nitrogen on shipboard. Thirdly, the large number of highly polymorphic microsatellite markers currently available provides substantially greater power for most analyses. And fourthly, microsatellites now offer lower data collection costs and higher throughput.

Applications of genetic data to stock mixtures extend beyond the management of adult fisheries. For example, recent studies have examined the stock composition of juvenile coho salmon in nearshore coastal areas (Teel et al. 2003) and migration patterns of chum salmon in the north Pacific (Seeb et al. 2004). Such studies will certainly become more prevalent as salmon recovery research focuses on how juvenile salmon uses estuarine and nearshore habitats, both temporally and spatially (Brodeur et al. 2000). In addition, highly variable microsatellite markers coupled with an expansion of analytical approaches (e.g., Hansen et al. 2001) offer great potential for estimating the stock origins of individual fish, even in complex mixtures. The identification of individuals will certainly become an important asset in the analyses of fish condition, growth and survival in estuarine and marine environments.

Forensic databases

In recent years, salmon fisheries enforcement has expanded from harvest on the high seas to protection of endangered species in freshwater habitats far from the ocean (Withler et al. 2004). Federal, state and tribal management agencies all have enforcement responsibilities that include identification of the origin of individual fish and groups of fish. These agencies must prosecute U.S. Endangered Species Act violations as well as state and tribal conservation-related crimes. There is an increasing interest in the forensic community in developing basin-wide databases that would help determine (at least probabilistically) the population-of-origin or ESU-of-origin of a given fish or fish tissue. The changing role of forensics and fisheries enforcement has created a convergence of management interests. For example, forensic efforts to characterise genetic diversity on a basin-wide level and identify ESU-specific markers will inevitably require some of the same standardisation measures that will be needed for combining population genetic data sets for other management purposes.

Marine mammal feeding ecology

Marine mammal trophic ecology is another management issue that has implications for basin-wide characterisation and genetic standardisation in salmon. Managers face a significant dilemma when federally protected marine mammal species prey on salmon populations that are listed under the U.S. Endangered Species Act [National Marine Fisheries Service (NMFS) 1997]. In an effort to quantify predation and determine which salmon species and stocks are involved, our group has undertaken collaboration with the National Marine Mammal Laboratory

(NMFS) to develop species-specific and ESU-specific markers within species. These markers have been used to analyse bones recovered from Pacific harbour seal (*Phoca vitulina richardsi*) scat (Purcell et al. 2004) and to estimate stock-of-origin for Chinook salmon (*Oncorhynchus tshawytscha*) (J. Rhydderch unpublished data).

These are examples of fundamental changes in the salmon genetics landscape in recent years. There has been an explosion of newly available microsatellite markers; the involvement of more laboratories and emerging issues in salmon recovery, harvest management, hatchery/wild interactions, marine mammal trophic ecology and forensics. These are the likely priorities that will shape the future of standardisation in ecological genetics.

Future of microsatellite standardisation in Pacific salmon

With the rapid growth of microsatellite analysis in Pacific salmon, there is already a need to begin standardisation and coordination of genetic data collection; however progress has been slow for lack of funding. As motivation for standardisation increases, it is important to anticipate specific research and management needs and to map out a collaborative interlaboratory effort to address those needs. This process was initiated in June 1999, and meetings in 2000 and 2001 pursued the goals set out in 1999. There was, however, little real action until the Pacific Salmon Commission came forth with significant funding in 2004. In the following sections, we summarise the considerations that emerged from data standardisation efforts up to that time. These issues are not necessarily specific to Pacific salmon nor to microsatellite data and should be expected to arise in a wide range of collaborative conservation research.

Four specific challenges to standardisation and suggested directions

Geographic scale of standardisation

In most cases, proximate efforts will be directed towards regional standardisation. Eventually, regionally standardised databases will expand or merge to become coastwide. Therefore, a certain amount of flexibility must be built in to regional standardisation activities. The broader the standardisation in terms of geography and concomitant genetic diversity, the more challenging the effort will become. Inevitably, greater genetic diversity is manifest as broader allele size ranges, perhaps overlapping other loci in multiplex electrophoresis and complicating genotyping of multiple loci (microsatellite loci are typically analysed as 'multiplex sets', groups of loci with non-overlapping

size ranges assayed simultaneously, see Olsen et al. 1996; Neff et al. 2000). As more distantly related populations are included in the standardisation, there is also an increased likelihood of encountering complex multimodal allele frequency distributions, null alleles, duplicated loci and other phenomena that may compromise the utility of selected loci. Expectations for range-wide, species-wide standardisation must be tempered by the levels of genetic variability likely to be encountered. Given the levels of mutation exhibited by many microsatellite loci, it may not be possible to standardise allele designations throughout species as diverse as, for example, *Oncorhynchus mykiss*, a large complex of many inland and coastal subspecies and life history forms including anadromous steelhead, resident rainbow trout and ancient redband (Behnke 1992). The recommendation is to consider the costs and benefits of standardising at various geographic scales. For example, driven by range-wide harvest management, the decision was made in Chinook salmon to standardise microsatellite markers coastwide. In contrast, management issues in steelhead and rainbow trout were more local and the cost and complexity of range-wide standardisation did not appear justified. Although current standardisation in *O. mykiss* includes three Pacific northwest subspecies, the focus is on the Columbia River basin. Broader geographic standardisation is always desirable, but may not always justify the cost.

Selection of loci

The selection of loci for standardisation is driven largely by historical inertia, i.e., laboratories favour loci used in the past and for which large data sets have accumulated. However, the initiation of a multi-laboratory standardisation requires re-evaluation of loci with focus on future needs. Highly polymorphic loci or loci with broad multimodal distributions are best avoided. Loci showing intermediate polymorphism should generally be favoured as a result of more reliable genotyping. Given that the more polymorphic loci will almost inevitably be more difficult to standardise, especially at broad geographic scales, they should probably be reserved primarily for fine-scale pedigree and genetic mapping studies where they are most valuable, at least on a per-locus basis. It is worth recognising, however, that any loss of resolution because of fewer alleles per locus can be made up through the use of more loci (Kalinowski 2002, 2004). The most important loci to avoid, irrespective of overall diversity, may be those that show imperfect repeat structures and other electrophoretic anomalies (see above). A final benefit to selecting a larger number of less polymorphic loci is that they tend to be smaller in size and therefore amplify more reliably from degraded DNA samples (e.g., from fish bones in

marine mammal scat; Purcell et al. 2004) or fish scales (Moran & Baker 2002). Most electrophoretic genotyping systems can resolve microsatellite alleles up to 500 bp or more; however, practical experience shows that loci less than 350 bp amplify more successfully from compromised DNA samples, as often must be used in ecological genetics, whether for salmon or other species, especially those of conservation concern. Even good quality DNA can exhibit 'upper allele drop out', in which the amplification of the larger allele in a heterozygous individual is diminished to the point that it is not identified, effectively creating a null allele. Upper allele drop out is directly related to increasing size range of alleles.

Many investigators favour tetranucleotide repeats; however, we find that they often exhibit less uniform mobility (e.g., variation in repeat unit increment) making bimodal distributions and imperfect repeats more challenging to standardise among laboratories. Plenty of dinucleotide markers are available for all salmonids and many other species that show very little stutter, an oft-cited liability of dinucleotide repeats. We therefore prefer dinucleotide repeats with compact distributions containing lots of alleles in a relatively small size range. This strategy tends to produce markers that amplify from degraded tissue, yet provide maximum information content while occupying little of the practical size range on the electrophoretic instrument (100–350 bp). If loci have narrow size ranges, it is not uncommon to simultaneously analyse two or three loci with each dye label in a multiplexed electrophoretic reaction. However, loci with a larger size range of alleles require that each dye label be dedicated to a single locus. This discussion assumes, of course, that single-base resolution is obtainable. Clearly, limited resolution would pre-empt these considerations in favour of tetranucleotide repeats.

Standardisation of allele designations

Based on the human forensic and diagnostic model, LaHood et al. (2002) proposed allele ladders as a method for interagency allele standardisation in salmon. A microsatellite allele ladder is a cocktail of PCR products pooled from multiple individuals and represents all or many of the alleles likely to be encountered in a given species for a particular locus. The diluted cocktail is distributed to other laboratories as a single control and cross-validation sample. Allele ladders provide the same verification of genotyping consistency that was achieved through distribution of reference tissues for standardising allozyme analysis, except that the ladders are more efficient because all alleles at a locus are PCR-amplified together and assayed in a single gel lane or a single injection through an electrophoretic capillary (i.e., the ladders

too can be analysed as a multiplex set because, like the loci themselves, they are non-overlapping, either in size or fluorescent label).

The allele ladder method as presented by LaHood et al. (2002) is also amenable to the addition of newly identified alleles, thus providing needed flexibility when the geographic scope of standardisation increases, and new interlaboratory collaborations are initiated (LaHood unpublished data). We encourage routine sequencing of representative alleles in studies using microsatellite loci; however, we believe that, along with tissue and DNA sample exchange, the allele ladders will be the most immediate and most useful tool in multi-laboratory collaborations.

Logistics of sharing unpublished data

The social landscape of salmon population genetic research in North America has also changed fundamentally, as genetic data have come to play a major role in natural resource policy decisions. Issues of intellectual propriety have become tempered by society's need to protect biodiversity. Resource management decisions often require expeditious distribution of unpublished population genetic data to state, tribal and federal agencies for independent analysis. Researchers in ecological and population genetics should expect requests for unpublished data. Specific requirements related to both distribution of results and data standardisation are already appearing in contracts with U.S. Fish and Wildlife Service and Bonneville Power Administration.

Sharing of unpublished data may involve various legal directives. For example, there are four sources of guidance concerning potential requirements to release genetic data under U.S. Federal laws:

- Freedom of Information Reform Act of 1986 (FOIA)
- Guidelines from the Department of Justice and the Office of Management and Budget
- Court rulings that required changes to earlier policies
- Executive Orders (e.g., Executive Order No. 12600, which requires notification to submitters of confidential business data prior to release).

The outcome of a particular request would depend on (i) the specific type of data requested (tissue samples, allele frequency data, preliminary analyses, draft reports); (ii) the type of organisation (state, Federal, university, tribal, individual) from which the original data were obtained and (iii) whether Federal funding supported the data collection. The FOIA is the most likely avenue for a request, although state laws might also be relevant.

The FOIA provides that any person has the right to request access to Federal agency records or information. Upon receiving a written request, Federal agen-

cies are required to disclose records, except for those that are exempted by any of the nine provisions in the statute. Three of the exemptions (trade secret/confidential information, pre-decisional deliberative material and law enforcement investigations) could potentially apply to requests for release of genetic data. Although it is possible that a FOIA exemption could be invoked to prevent the release of genetic data, there have been no court cases, to date, interpreting the FOIA as applied to genetic data. It remains an uncertain issue that is relevant to shared standardised data sets and conditional use agreements.

Conclusions

There will be increasing need in the future to collect genetic data in a way that is conducive to integration of individual studies – indeed, management is insisting on it. Current efforts can benefit both technically, logistically and conceptually from an historical perspective that draws on experience with allozymes. Genetic technology is evolving so rapidly that it is difficult to predict the long-term importance of microsatellite analysis. It seems likely, however, that for at least the next 10 years or so, microsatellites will play a pivotal role in ecological genetics. Although numerous difficulties remain in the standardisation of microsatellites for Pacific salmon and other species, the pay-off is substantial in terms of the added utility of combined data to address a broad array of new problems not originally identified in the individual studies.

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