Exxon Valdez Oil Spill
Restoration Project Final Report

Genetic Discrimination of Prince William Sound Herring Populations

Restoration Project 97165
Final Report

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Note: Peer review comments have not been addressed in this final report.

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Study History: This multi-year study was initiated in FY94 as Restoration Project 94165. However, a return failure of herring in Prince William Sound in 1994 delayed implementation until FY95. During the first two years (FY95, FY96) new molecular markers were developed and tested for population genetic analyses. In addition, herring samples were collected from spawning aggregations within Prince William Sound (4 sites), Kodiak Island (1 site), Togiak Bay (1 site), and Norton Sound (1 site). During the final two years (FY97, FY98) samples were genotyped and genetic data were analyzed. Preliminary results were reported to the *Exxon Valdez* Oil Spill (EVOS) Trustee Council and EVOS project reviewers in a poster session at the January 1996 EVOS workshop in Anchorage. A synthesis of the herring genetic data was reported to the EVOS peer reviewers in February 1998. Four reports were submitted by consulting scientists detailing the development and application of mtDNA and microsatellite markers. One progress report was submitted by the principal investigators in 1996 under the title Genetic Discrimination of Prince William Sound Herring Populations.

Abstract: We examined spatial and temporal patterns of genetic variation at five microsatellite loci and one mtDNA gene in seven spawning aggregations of Pacific herring (*Clupea harengus*) sampled in 1995 and 1996. Sample locations included Prince William Sound (4 sites) and Kodiak Island (1 site) in the Gulf of Alaska, and Togiak Bay (1 site) and Norton Sound (1 site) in the Bering Sea. An analysis of molecular variance revealed a marked genetic discontinuity between herring in the Bering Sea and Gulf of Alaska. The estimates of genetic differentiation between populations from the two sea basins (F_{BT}) were significant ($P<0.001$) and ranged from 0.169 for mtDNA to 0.209 for microsatellites. The estimates of genetic differentiation within sea basins (F_{SB}) were 0.013 for mtDNA and 0.030 for microsatellites and were significant ($P<0.001$). The microsatellites also showed significant ($P<0.001$) temporal variation within the Gulf of Alaska. The estimates of genetic variation between years at each sample location (F_{SL}) were 0.011 for microsatellites and 0.012 for mtDNA. The temporal shifts in genetic structure may reflect large inter-annual variation in reproductive success and hamper the use of genetic markers for discrete stock management of herring in Prince William Sound.

Key Words: *Clupea harengus*, DNA, *Exxon Valdez* oil spill, genetics, Pacific herring, populations, Prince William Sound, restoration.

Project Data: *Description of data* – Two data sets were developed during this project: (1) microsatellite and mtDNA sequence data for primer development; and (2) microsatellite genotype data and mtDNA haplotype data for each individual in each population. In addition, historic allozyme data was obtained from an earlier study. *Format* – These data are in text files in MS Word and spreadsheet format in MS Excel, Office 97. *Custodian* – Contact Sue Merkouris at the Alaska Department of Fish and Game, Genetics Laboratory, 333 Raspberry Road, Anchorage, Alaska 99518 (work phone: (907) 267-2138, fax: (907) 267-2442 or e-mail at sue_merkouris@fishgame.state.ak.us). *Availability* – Copies of all text files are available via e-mail or on 3.5 inch disk for the cost of duplication. The cloned microsatellite sequence data can

be obtained by searching for "Pacific herring" in GenBank on the internet (http://www.ncbi.nlm.nih.gov/Entrez/nucleotide.html).

Citation:

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Appendix B: *Published manuscript* – O'Connell, M., M.C. Dillon, J.M. Wright, P. Bentzen, S. Merkouris, and J. Seeb. 1998. Genetic structuring among Alaskan Pacific herring populations identified using microsatellite variation. Journal of Fish Biology. 53:150-163.

Appendix C: *Final report* – Wright J. M. and M.C. Dillon. 1997. Temporal stability of microsatellite markers in Prince William Sound herring populations.

Appendix D: *Annual report* – Bentzen, P. 1997. Development of mitochondrial DNA markers and use to screen Prince William Sound herring populations for genetic differentiation.

Appendix E: *Final report* – Bentzen P., J. Olsen, J. Britt, and K. Hughes. 1998. Molecular genetic polymorphism in Alaskan herring (*Clupea pallasi*) and its implications for population structure.

EXECUTIVE SUMMARY

Scope of Study

- *New microsatellite and mtDNA markers were developed and used to examine genetic variation in seven putative populations of Pacific herring from the Gulf of Alaska and Bering Sea.*

We examined spatial and temporal patterns of genetic variation in Pacific herring from seven locations within and adjacent to Prince William Sound (PWS) in 1995 and 1996. Four collection sites were chosen within PWS to maximize the potential genetic differentiation among temporally and spatially isolated spawning aggregations. We targeted Rocky Bay and Port Chalmers, early-spawning isolates from Montague Island in southcentral PWS; St. Matthews Bay, a late-spawning isolate in southeast PWS; and Fish Bay, a late-spawning isolate in northeast PWS. Three collection sites outside PWS were chosen to test for genetic differentiation on a broad geographic scale and provide spatial context within which to evaluate population structure of PWS herring. We sampled Kodiak Island (1 site) because its populations are thought to share an ancestral tie with PWS populations (J. Wilcock, Alaska Department of Fish and Game, personal communication). We sampled Togiak Bay and Norton Sound because Bering Sea populations are known to be genetically isolated from Gulf of Alaska stocks. Single collections were made at all spawning sites in 1995 and 1996 with the exception of the Bering Sea populations which were sampled in 1991 and 1995.

Two classes of DNA markers, microsatellites and mtDNA, were selected to test for spatial and temporal patterns of genetic variation in Pacific herring. Extensive marker development and testing was required prior to population screening. Primers for Polymerase Chain Reaction (PCR) were developed to amplify five di-nucleotide (gt_n) microsatellites isolated from herring DNA using standard cloning protocol and DNA sequencing technology. Primers developed for amplification of a 2 kilobase fragment of mtDNA in chinook and chum salmon were redesigned for herring to improve PCR efficiency. Twelve enzymes were tested for restriction fragment length polymorphism (RFLP) analysis of the mtDNA gene of which three were selected based on restriction site polymorphism in one or more populations.

After data analysis was complete it became apparent that more microsatellite loci (especially those with tetra-nucleotide repeats) could help to better elucidate the evolutionary forces affecting population structure of herring in Prince William Sound, the Gulf of Alaska and Bering Sea (see suggestions for further research below). Therefore, this project funded development of primer sequences for 20 new microsatellites for herring. These loci will consist of primarily tetra-nucleotide repeats and will be used by the Alaska Department of Fish and Game in future genetic studies of Pacific herring.

Spatial patterns of genetic variation

- *Both marker types confirmed a large genetic discontinuity between Pacific herring from the Gulf of Alaska and Bering Sea.*

The DNA data confirmed results of an earlier allozyme study that concluded the Alaska Peninsula is a major zoogeographic boundary for Pacific herring, restricting gene flow between populations in the Gulf of Alaska and Bering Sea. An analysis of molecular variation (AMOVA) revealed a substantial proportion of the total genetic variance in herring was due to differences

among populations from the two sea basins (F_{BT}). For microsatellites, the estimates of F_{BT} were 0.023 (based on mean variance in allele frequency) and 0.209 (based on mutational distance among alleles). The later estimate is believed to best reflect the evolution of microsatellite variation at this scale and is consistent with results from other markers. The estimates of F_{BT} were 0.169 for mtDNA and 0.241 (from historic data) for allozymes. All estimates of F_{BT} were highly significant ($P < 0.001$).

- *Microsatellites revealed significant variation between sample locations within Prince William Sound, the Gulf of Alaska, and the Bering Sea in 1995 and 1996.*

Estimates of the proportion of the total genetic variation due to differences among populations within sea basins (F_{SB}) was an order of magnitude smaller than F_{BT}. The estimates of F_{SB} were 0.013 for mtDNA and 0.030 (based on mutational distance among alleles) for microsatellites and were highly significant ($P < 0.001$). The estimate of F_{SB} was 0.003 (from historic data) for allozymes and was not significant ($P = 0.488$). For microsatellites, allelic frequency variation among samples collected in both years was significant on all spatial scales including within the Bering Sea, within the Gulf of Alaska, and within Prince William Sound. For mtDNA, haplotype frequency variation was not significant among samples collected in the Bering Sea, or among samples collected in the Gulf of Alaska in 1995 but was significant among samples collected in Prince William Sound in 1996.

Temporal patterns of genetic variation

- *Microsatellites revealed significant inter-annual variation at locations sampled in successive years within Prince William Sound.*

Comparisons among samples taken in the same locations but in different years yielded an important result: the magnitude of genetic variation among sampling years within locations was equal to or greater than the magnitude of variation among locations within sea basins. An AMOVA was conducted to estimate the proportion of genetic differentiation due to temporal versus spatial factors in spawning aggregations from the Gulf of Alaska. This analysis revealed that the proportion of genetic variance that occurred among samples (years) within locations was similar for both microsatellites ($F_{SL} = 0.011$) and mtDNA ($F_{SL} = 0.012$), but only significant for microsatellites ($P < 0.001$). By contrast, estimates of the proportion of genetic variance associated with variation among locations after removing the temporal effect were actually negative for both markers ($F_{LT} = -0.003$) and of course, not significant ($P \geq 0.88$). Thus, even though the DNA data revealed significant differences among population samples on geographic scales as fine as within Prince William Sound, these differences were not reproducible from year to year. Indeed, probability tests showed significant ($P < 0.001$) allele frequency heterogeneity for at least one microsatellite locus (range = 1-3) for paired samples drawn from the same locations in different years in Prince William Sound (except Rocky Bay). These samples did not group together in dendrograms based on genetic distance.

Interpretation of results and suggestions for further research

- *The apparent temporal instability in genetic differentiation may hamper use of genetic markers for discrete stock management.*

This study yielded two important results. First, the DNA data indicated a marked genetic discontinuity between herring in the Bering Sea and Gulf of Alaska. Genetic discontinuities of this sort are uncommon in continuously distributed pelagic marine species, but a number of similar examples have been encountered, particularly around the Florida peninsula and other known zoogeographic boundaries. Second, the DNA data provided no evidence of stable differentiation among populations within sea basins on spatial scales of up to ~700 km. Rather, the DNA data suggested that temporal variation among spawning aggregations dominates genetic variability on these spatial scales. Such variation has been observed in a number of other pelagic marine fishes, including California sardine (*Sardinops sagax caeruleus*) and northern anchovy (*Engraulis mordax*). The lack of stable differences in allele frequencies among herring spawning aggregations do not disprove the existence of demographically independent local spawning stocks, but nor do they provide any positive evidence in support of this hypothesis. Finally, the shifting patterns of genetic structure may hamper the use of genetic markers for discrete stock management on small spatial scales such as Prince William Sound.

- *Future studies should examine temporal variation more closely by using additional genetic markers and refining the sampling scheme.*

Further work with DNA markers is warranted for at least two reasons. First, the striking differentiation between the Bering Sea and Gulf of Alaska, and the exact position and the nature of the boundary between these two genetic races deserves further investigation. Second, different sampling protocols might yield useful insights into the processes driving genetic differentiation on small - medium geographic scales.

INTRODUCTION

Pacific herring (*Clupea harengus*) play an important role in the ecosystem, economy and native cultures of Prince William Sound (PWS). The timing of the *Exxon Valdez* oil spill (EVOS) overlapped the annual spring migration to nearshore staging areas of herring spawners. Over 40% of the herring spawning, staging, and egg deposition areas and over 90% of the documented summer rearing and feeding areas were lightly to heavily oiled prior to the spawning events. As a result, herring encountered oil during each of their four life stages in 1989 and, to some extent, in 1990. Adult herring traversed oil sheens while traveling northward and eastward. Eggs were deposited on oiled shorelines and were exposed to sheen through tidal action while incubating. Larvae that hatched contained lipophilic petroleum hydrocarbons in their yolk sacs and encountered sheen near the surface while in their most sensitive state. Post-larval or juvenile herring swam through and remained near lightly to heavily oiled shorelines, regularly encountering sheen, and dissolved oil components through the summer while feeding in shallow nearshore bays and passes.

The Prince William Sound herring population began to decline in 1992. By 1994 the number of spawners fell below the management objective, forcing cancellation of the commercial fishery. The fishery remained closed for four years (1994-1997). Preliminary pathology results suggested viral hemorrhagic septicemia (VHS) as a potential source of mortality and stress, however this was not conclusive (Meyers et al. 1994). In 1994, the Alaska Department of Fish and Game began a recovery effort that included pathology, genetics, early life history, and oceanographic investigations. The Department drafted a stock model (Brown and Wilcock 1994) to provide a basis for restoration management. However, the stock model included several assumptions about the population structure of Prince William Sound spawning groups. Genetic homogeneity of herring stocks within PWS and no recruitment to those stocks from outside of the Sound are two of the assumptions this project was designed to evaluate.

Incorporating genetically-derived population structure is crucial to the success of any fisheries or restoration program. Consistent exploitation of mixed populations has to lead to the demise of the least productive stocks (Schweigert 1993). Unfortunately, defining the population structure of herring has been particularly difficult. There is evidence that herring home (Wheeler and Winters 1984), but straying may also be substantial. Morphological and meristic differentiation of herring from discrete geographic regions has been used as evidence for the existence of genetically distinct populations, but much of this variation may be environmentally mediated and has not been confirmed with genetic data (Safford and Booke 1992; King 1985; Burkey 1986).

Allozyme electrophoresis has proven to be a useful tool for delineating the population structure of herring over broad geographic regions (Grant 1984; Grant and Utter 1984) and between spawning populations within the same area that are temporally isolated (Kornfield et al. 1982). Allozymes define two distinct races of Pacific herring (Asian/Bering Sea and eastern North Pacific), with further subdivision between Gulf of Alaska and more southerly North Pacific stocks (Grant and Utter 1984). Also, allozyme markers describe genetic divergence among local spawning populations of Pacific herring in the vicinity of northern Japan (Kobayashi et al. 1990) and among genetically distinct fjord populations in Norway (Jorstad et al. 1994).

Additional techniques to study the structure of natural populations have become available in recent years as a result of advances in molecular biology. Restriction fragment length

polymorphism (RFLP) analysis of mitochondrial DNA (mtDNA) provided some evidence of genetic differentiation within Atlantic and Pacific herring (Kornfield and Bogdanowicz 1987; Schweigert and Withler 1990; Dahle and Eriksen 1990); however the utility of these techniques to detect fine genetic structure in Pacific herring from the Gulf of Alaska has not been fully assessed. Nuclear DNA microsatellite markers are a new class of markers with the potential of being useful for investigation of fine population structure (e.g., Taylor and Bentzen 1993; Bentzen et al. 1994). Nuclear and mitochondrial loci evolve in response to different pressures and reflect differing patterns of relationships among populations. In this study we pursued a combination of both mitochondrial and microsatellite approaches to more accurately define the stock structure of herring from the EVOS-affected area. These data may also be used to estimate the population composition of non-spawning aggregations contributing to fall fisheries in Prince William Sound.

The goal of this project was to improve the accuracy of current stock assessment methods, thus improving resource management. Improved accuracy of stock distribution information will allow fishery managers to make fine adjustments of fishing quotas to harvest the maximum available surpluses with the lowest possible risk of over harvest, damage to the resource, or economic loss to the fishing industry.

OBJECTIVES/RESULTS

Our overall objective was to provide a genetic basis for the stock model used by Alaska Department of Fish and Game to manage and restore the depleted herring resource in Prince William Sound. We tested for genetic heterogeneity among spawning aggregations of Pacific herring within Prince William Sound, adjacent to Prince William Sound in the Gulf of Alaska and in the Bering Sea, and between year classes within and adjacent to the Sound. A brief summary of the results as they apply to each objective is provided here. More detailed results are provided in the appendices

Objective 1:

Survey population samples using both mitochondrial and nuclear DNA approaches. Techniques included RFLP analysis of mtDNA and Polymerase Chain Reaction (PCR) fragment analysis of microsatellite loci.

Result:

Five new microsatellites and one mtDNA gene were used to examine genetic variation in seven putative populations of Pacific herring from the Gulf of Alaska and Bering Sea.

Objective 2:

Evaluate the null hypothesis that a single panmictic population of herring exists in Prince William Sound. The study included four putative population samples from both spatial and temporal isolates within the Sound.

Result:

Microsatellites revealed significant variation between sample locations within Prince William Sound, the Gulf of Alaska, and the Bering Sea in 1995 and 1996. Microsatellites also revealed significant inter-annual variation at locations sampled in successive years within Prince William Sound. The degree of spatial and temporal variation in mtDNA was similar to microsatellites but not significant.

Objective 3:

Evaluate the structure of Prince William Sound herring populations within the context of the structure of adjacent spawning aggregates, including comparisons from across the known genetic barrier of the Alaska Peninsula

Result:

Both marker types confirmed that a substantial component of genetic variation in Pacific herring is due spatial isolation in the Gulf of Alaska and Bering Sea. This variation is much greater than that found within either sea basin.

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LITERATURE CITED

Bentzen, P., D. B. Morris, and J. M. Wright. 1994. Development and use of variable number tandem repeat markers for population and aquacultural genetics of salmonids. Pages 85-90. *In*: L. K. Park, P. Moran, and R. S. Waples (eds.) Application of DNA Technology to the Management of Pacific Salmon. NOAA Technical Memorandum NMFS-NWFSC-17.

Brown, E. D., and J. A. Wilcock. 1994. A stock model for Pacific herring in Prince William Sound. Draft report available from the Cordova Area Office, Alaska Department of Fish and Game, December 9, 1994. 15pp.

Burkey, Jr., C. 1986. Population structure of Pacific herring (*Clupea harengus pallasi*) in eastern Prince William Sound, Alaska. M. S. Thesis. University of Alaska, Fairbanks, Juneau Center for Fisheries and Ocean Studies, 11120 Glacier Hwy., Juneau, AK, 99801. 117 pp.

Dahle, G., and A. G. Eriksen. 1990. Spring and autumn spawners of herring (*Clupea haren*gus) in the North Sea, Skagerrak and Kattegat; population genetic analysis. Fish Res. 9:131-141.

Grant, W. S. 1984. Biochemical population genetics of Atlantic herring, *Clupea harengus*. Copeia (2):357-364.

Grant, W.S., and Utter, F.M. 1984. Biochemical population genetics of Pacific herring (*Clupea pallasi*). Can. J. Fish. Aquat. Sci. 41:856-864.

Hedgecock, D. 1994. Temporal and spatial genetic structure of marine animal populations in the California current. CalCOFI Rep. 35:73-81.

Jorstad, K. E., G. Dahle, and O. I. Paulsen. 1994. Genetic comparison between Pacific herring (*Clupea pallasi*) and a Norwegian fjord stock of Atlantic herring (*Clupea harengus*). Can. J. Fish. Aquat. Sci. 51 (Suppl. 1):233-0239.

King, D. P. F. 1985. Enzyme heterozygosity associated with anatomical character variance and growth in the herring (*Clupea harengus* L.). Heredity 54:289-296.

Kobayashi, T., M. Iwata, and K. Numachi. 1990. Genetic divergence among local spawning populations of Pacific herring in the vicinity of northern Japan. Nip. Suis. Gak.-Bull. Jap. Soc. Sci. Fish. 56:1045-1052.

Kornfield, I., and S. M. Bogdanowicz. 1987. Differentiation of mitochondrial DNA in Atlantic herring, *Clupea harengus*. U. S. Fish. Bull. 85:561-568.

Kornfield, I., B. D. Sidell, and P. S. Gagnon. 1982. Stock identification in Atlantic herring (*Clupea harengus harengus*): genetic evidence for discrete fall and spring spawning populations. Can. J. Fish. Aquat. Sci. 39:1610-1621.

Meyers, T. R., S. Short, K. Lipson, W. N. Batts, J. R. Winton, J. Wilcock, and E. Brown. 1994. Association of viral hemorrhagic septicemia virus with epizootic hemorrhages of the skin

in Pacific herring *Clupea harengus pallasi* from Prince William Sound and Kodiak Island, Alaska. USA, Dis. Aquat. Org.19:27-37.

Safford, S. E., and H. Booke. 1992. Lack of biochemical genetic and morphometric evidence for discrete stocks of northwest Atlantic herring *Clupea harengus harengus*. Fish. Bull. 90:201-210.

Schweigert, J. F., and R. E. Withler. 1990. Genetic differentiation of Pacific herring based on enzyme electrophoresis and mitochondrial DNA analysis. Amer. Fish. Soc. Symp. 7:459-469.

Schweigert, J. F. 1993. Evaluation of harvesting policies for the management of Pacific herring stocks, *Clupea pallasi*, in British Columbia. Pages 167-190. In: G. Kruse, D. M. Eggers, R. J. Marasco, C. Pautzke, and T. R. Quinn (eds.) Management Strategies For Exploited Fish Populations, Alaska Sea Grant College Program, University of Alaska, Fairbanks, Rep. No. 93-

Taylor, E. B., and P. Bentzen. 1993. Molecular genetic evidence for reproductive isolation between sympatric populations of smelt Osmerus in Late Utopia, south-western New Brunswick, Canada. Mol. Ecol. 2:345-357.

Wheeler, J. P., and G. H. Winters. 1984. Homing of Atlantic herring (*Clupea harengus*) in Newfoundland waters as indicated by tagging data. Can. J. Fish. Aquat. Sci. 41:108-117.

APPENDICES

APPENDIX A

Table 2 Number of alleles in cross-species amplification. M and – indicate multilocus banding pattern and absence of amplification, respectively

		Locus					
	n	Cop3–4	Cop4–1	Cop5	Cop10	Cop24–3	Cop111
Cancridae							
Cancer magister	2	3	–	–	–	1	–
Majidae							
Chionoecetes bairdi	10	14	9	M	8	10	5
Hyas araneus	5	2	7	1	1	1	1
Hyas coarctatus	5	10	6	1	1	1	1
Hyas lyratus	8	7	5	1	1	1	–
Oregonia gracilis	4	6	4	1	–	–	1
Pugettia gracilis	4	–	3	1	–	1	–

room temperature for 1–3 days. Loci Cop3–4, Cop5, and Cop10 consisted of a mixture of mono-, di-, tri- or tetranucleotide repeats. Among the six primer pairs, five produced patterns as expected from single loci inherited in a mendelian fashion. However, primers designed for locus Cop5 yielded a multiple banding pattern with a variable number of bands per individual ranging from nine to two in 55 individuals with an average of 5.7 (data not shown).

The amplification of the six loci in seven crab species revealed DNA polymorphisms at one or more loci for every species (Table 2). The variability at microsatellite loci observed in *C. opilio* compared to that observed in other species was generally higher or approximately equal. However, locus Cop10 was not very polymorphic in *C. opilio*, but was highly variable in its close relative *C. bairdi*.

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References

Ali S, Muller CR, Epplen JT (1986) DNA fingerprinting by oligonucleotide probes specific for simple repeats. *Human Genetics*, **74**, 239–243.

Beroud C, Antignac C, Jeanpierre C, Junien C (1990) Un programme informatique pour la recherche d'amorces pour l'amplification par PCR. *Médecine/Sciences*, **6**, 901–903.

Bowcock AM, Osborne-Lawrence S, Barnes R *et al.* (1993) Microsatellite polymorphism linkage map of human chromosome 13q. *Genomics*, **15**, 376–386.

Dower WJ, Miller JF, Ragsdale CW (1988) High efficiency transformation of *E. coli* by high voltage electroporation. *Nucleic Acids Research*, **16** (13), 6127–6145.

Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press, New York.

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain terminating inhibitors. *Proceedings of the National Academy of Sciences of the United States of America*, **74**, 5463–5467.

Development of primers for polymorphic microsatellite loci in the Pacific herring (*Clupea harengus pallasi*)

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Pacific herring (*Clupea harengus pallasi*) is a vital component in the diet of many species including marine mammals, fish and shore birds and also represents a valuable commercial fishery. In 1989 the Exxon Valdez released 41 million litres of crude oil within Prince William Sound, Alaska and the surrounding areas. Large areas of the Prince William Sound herring spawning and feeding areas were polluted with oil prior to the annual spawning event. This has led to severe declines in the size of the annual spawning event (< 33% of prespill predictions) (Brown *et al.* 1996).

To best conserve this biological resource, an appreciation of the levels of genetic variability and divergence among populations is required. However, past genetic studies using allozymes have revealed low levels of variation and limited discrimination within the Gulf of Alaska (e.g. Grant & Utter 1984). Microsatellite markers have displayed genetic variation in many species which have revealed little, if any, variation using other markers (e.g. Taylor *et al.* 1994). Therefore, primers for microsatellite loci were developed for Pacific herring and the levels of variability at six loci are described.

Table 1 Estimates of variability at the five polymorphic loci investigated in detail

Locus	Primer sequences (5'-3')*	Number of alleles	Size range (bp)	Heterozygosity	Annealing (°C)	Sequenced allele repeat number	Sample size (*n*)
Cha17	GAGACTTACTCTCATCGTCC GCACAGTAGATTGGTTCCAC	36	102–182	0.926	57	$GT_{(24)}$	187
Cha20	GTGCTAATAGCGGCTGCTG TTGTGGCTTTGCTAAGTGAG	20	100–144	0.874	57	$GT_{(20)}$	199
Cha34	CCTATGCTATCCTAACGATGG GCTATGACCATGATTACGG	21	75–127	0.895	57	$GT_{(30)}$	97
Cha63	TGCCTGCTGAAGACTTCC CCCCTAAATGTGTTCTTTTAGC	26	130–180	0.911	57	$GT_{(29)}$	199
Cha113	CTCTTCCATTTCGCTACGG GCAGTCTGACTTTACAATGA	24	100–150	0.888	52	$GT_{(24)}$	196
Cha123	GGGACGACCAGGAGTG AAATATAGTTTTATGATTGGCT	38	150–226	0.932	52	$GT_{(26)}$	195
Mean		29		0.906			195

*The primer sequences have been submitted to GenBank and are available under the following accession numbers: *Cha*17, AF019993; *Cha*20, AF019989; *Cha*34, AF019991; *Cha*63, AF019987; *Cha*113, AF019994; *Cha*123, AF019992.

Approximately 60 µg of Pacific herring DNA was digested with *RsaI*, *PalI* and *HincII*. The 300–700 bp size fraction was recovered and cloned into dephosphorylated pUC18 (Pharmacia) digested with *SmaI*. The ligation products were then used to transform MAX EFFICIENCY DH5α (GIBCO) cells. Approximately 10000 colonies were lifted using Hybond-N (Amersham) and fixed according to the supplier's recommendations. A $GT_{(15)}$ probe, labelled with [γ-^{32}P]-ATP using T4 polynucleotide kinase (PNK) (New England Biolabs), was added to the hybridization mixture and the reaction was allowed to proceed overnight at 62 °C (see O'Connell *et al.* 1996 for details). The membranes were washed twice at room temperature in 2× SSC/0.2% SDS and exposed to X-ray film (X-OMAT-R; KODAK) for 6 h at –80 °C. Over 1000 clones hybridized to the oligonucleotide probe.

One hundred and eighty-five individual clones were sequenced in both directions. Primers were designed for nine loci, six of which amplified consistently and showed products within the expected size range (Table 1).

DNA was extracted from 200 individuals collected from sites within Prince William Sound, Alaska. Muscle tissue was incubated for 2 h at 55 °C in a proteinase K/SDS solution (100 µL). DNA was precipitated with the addition of an equal volume of isopropanol (100 µL), dried for 10 min and resuspended in TE (pH 8.0). Primer labelling was carried out by incubating with PNK and [γ^{32}P]-ATP (Dupont) for at least 30 min at 37 °C. PCR amplification was performed in a 5 µL volume using a PTC-100 MJ thermal cycler (MJ Research Inc.). Each reaction contained ≈ 20 ng of template DNA, 100 µM of each dNTP, 1 mM $MgCl_2$, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 0.5 µM of each primer, 0.01% gelatin, 0.1% Tween-20 and 0.5 units of *Taq* polymerase (Promega). The annealing temperature for *Cha113* and *Cha123* was 52 °C. For *Cha70* an annealing temperature of 55 °C was used. The remaining loci were amplified at an annealing temperature of 57 °C. For the first five cycles, samples were denatured at 94 °C, primers were annealed at their specific optima and primer extension was carried out at 72 °C. For the remaining 35 cycles, the denaturation temperature was reduced to 90 °C. All steps were carried out for 20 s.

Loading dye (Pharmacia Sequencing kit) (5 µL) was added to the samples on completion of PCR analysis. The PCR products were then denatured at 94 °C for 10–20 min and 3 µL of the sample was loaded on an 8% denaturing gel. Gels were run at ≈ 60mA for 2.5–3 h. The gels were then fixed, dried for 1 h and exposed to X-OMAT AR film from between 18 h to 4 days without intensifying screens at room temperature. All gels were run with three sets of size standards (M13 sequence) and specific samples were reloaded across gels to increase the accuracy of scoring.

The numbers of alleles observed per locus and expected heterozygosity (Nei 1987) was very high in all cases (Table 1) and suggests that these loci will prove useful in discriminating among closely related Pacific herring populations. Using identical PCR conditions, the primers were also tested for cross-species amplification products in the related Atlantic herring (*Clupea harengus harengus*), menhaden (*Brevoortia tyrannus*) and cape anchovy (*Engraulis capensis*). These tests revealed no amplification products except in Atlantic herring. The loci revealed similar high levels of variability in Atlantic herring (observed heterozygosity estimates ranged from 0.77 at *Cha63* to 0.92 at *Cha17*) within the same size range observed for Pacific herring (P. Shaw, personal communication).

Acknowledgements

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References

Brown ED, Norcross BL, Short JW (1996) An introduction to studies on the effects of the Exxon Valdes oil spill on early life-history stages of pacific herring (*Clupea pallasi*) in Prince William Sound Alaska. *Canadian Journal of Fisheries and Aquatic Sciences*, 53, 2337–2342.

Grant WS, Utter FM (1984) Biochemical population genetics of Pacific herring (*Clupea pallasi*). *Canadian Journal of Fisheries and Aquatic Sciences*, **41**, 856–864.

Nei M (1987) *Molecular Evolutionary Genetics*. Columbia University Press, New York.

O'Connell M, Wright JM, Farid A (1996) Development of PCR primers for nine polymorphic American mink, *Mustela vison*, microsatellite loci. *Molecular Ecology*, **5**, 311–312.

Taylor AC, Sherwin WB, Wayne RK (1994) Genetic variation of simple sequence loci in a bottlenecked species: the decline of the hairy-nosed wombat (*Lasiorhinus krefftii*). *Molecular Ecology*, 3, 277–290.

Isolation and characterization of microsatellite markers in the *Ixodes ricinus* complex (Acari: Ixodidae)

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The European tick *Ixodes ricinus* is a species of great medical and veterinary importance, serving as the vector of tick-borne encephalitis, rickettsiosis, piroplasmosis and Lyme disease. Nonetheless, its migratory capabilities are poorly understood. Indirect methods based on genetic markers show great potential utility to evaluate the historical levels and patterns of gene flow that have given rise to the observed pattern of genetic variation (Slatkin 1985). Allozyme markers have revealed little polymorphism in *I. ricinus* (Healy 1979a,b; Delaye *et al.* 1997). To obtain more information, we considered microsatellites because they have been useful in many species including insect vectors such as *Anopheles gambiae* (Lanzaro *et al.* 1995; Lehmann *et al.* 1996). Moreover, studies (see, for example, Hughes & Queller 1993) show that highly polymorphic microsatellites have been found in species with little allozymic variation polymorphism. Here we report the development of primers for PCR amplification of six microsatellite loci in the tick *I. ricinus*.

Two genomic libraries were constructed successively as described in Estoup *et al.* (1993). For each library, 30 μg of genomic DNA from 20 individuals was restricted with the enzyme *Sau*3AI. The fragments were separated on a 1% low-melting-point agarose gel and the 400–600 fragments isolated and ligated into a pBluescript vector (Stratagene). The ligation products were transformed into XL1-Blue MRF' Supercompetents cells (Stratagene) and the resulting colonies were blotted on Hybond-N+ membranes which were hybridized with a mixture of two probes $(CA)_n$ and $(GA)_n$. Two thousand clones from each library gave four and 34 positively hybridizing clones, respectively, of which 29 clones were sequenced. The majority of the microsatellites had no more than 10 repeats. Most had a mononucleotide repeat (A or C) adjacent to the dinucleotide (CA or GA) repeat region. After removing almost all of the clones which either had associated mononucleotide repeats or which were truncated by the end of the insert, 12 loci were subsequently targeted for PCR amplification. The primers were designed using OSP software (L. Hiller Washington, URL: infobiogen.fr). Six gave clear PCR products of the expected size. The PCR primers designed for the six loci are described in Table 1. The GenBank accession numbers for the nucleotide sequences of these six microsatellite loci are AFO24667 to AFO24671 and AFO25851.

The genomic DNA for genotyping was prepared using a phenol–isopropanol extraction method. Initially, one primer from each pair was end-labelled with T4 polynucleotide kinase and [γ-^{33}P]-ATP. The PCR amplifications were carried out in 20 μL of a mixture containing 10–50 ng of template DNA, 1× *Taq* polymerase buffer, $MgCl_2$ (concentration in Table 1), 200 μM each dNTP, 3 pmol of each primer and 0.5 U of *Taq* polymerase (Goldstar, Eurogentec SA). Initial denaturation was 4 min at 94 °C followed by 30 cycles (94 °C for 30 s, 30 s at annealing temperature as specified in Table 1 and 72 °C for 30 s) and 10 min at 72 °C in a thermocycler (Crocodile II, Appligene). PCR products were resolved in 5% acrylamide–bisacrylamide and 8 M urea sequencing gels. Clones already sequenced served for size control.

In a sample of 50 individuals, five of these six loci were polymorphic, with more than 16 alleles identified in each of three loci leading to high expected heterozygosities (Table 1). We note that the least polymorphic locus, IR27, has the most often interrupted repeats.

We have begun evaluating these primers on *I. dammini*, the Lyme disease vector prevalent in northeastern and upper midwestern North America. With two individuals and the PCR conditions used in *I. ricinus*, we succeeded in amplifying five out of six loci (IR8, IR18, IR27, IR32 and IR39). These first tests are encouraging in the possibility of using these primer sets with other members of the *I. ricinus* complex.

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References

Delaye C, Beati L, Aeschlimann A, Renaud F, De Meeûs T (1997) Population genetic structure of *Ixodes ricinus* in Switzerland from allozymic data: no evidence of divergence between nearby sites. *International Journal for Parasitology*, 27, 769–773.

APPENDIX B

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Genetic structuring among Alaskan Pacific herring populations identified using microsatellite variation

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Five highly variable microsatellite loci were used to investigate population structuring in Pacific herring *Clupea pallasi* collected from Kodiak Island, two sites in the Bering Sea and four sites within Prince William Sound, Alaska. All loci revealed high levels of variability with heterozygosity estimates ranging from 86 to 97% (mean heterozygosity: 89%). The variation was structured significantly among sites suggesting that the samples investigated were genetically distinct from each other. Genetic divergence was greatest between populations from the Bering Sea and those from Prince William Sound. The Kodiak Island and Point Chalmers samples appeared to be distinct from the Prince William Sound and Bering Sea populations. The observed genetic distance relationships among samples could be explained largely in terms of geographical separation.

Key words: Pacific herring; *Clupea pallasi*; microsatellite variation.

INTRODUCTION

Pacific herring *Clupea pallasi* Valenciennes are commercially important throughout much of the Pacific Rim. As such, a description of the population structure has remained a central focus of herring research and management in both North America and Asia (Grant & Utter, 1984; Kobayashi *et al.*, 1990; Rowell *et al.*, 1990; Schweigert & Withler, 1990). Knowledge of genetic population structure is crucial to the success of long-term fisheries conservation because consistent exploitation of mixed populations often leads to the demise of the least productive stocks (Schweigert, 1993).

Our interest in the population structure of herring was stimulated by the *Exxon Valdez* oil spill in Prince William Sound, Alaska. The 41 million-litre spill substantially influenced populations of Pacific herring inhabiting Prince William Sound in the spring of 1989. The timing of the *Exxon Valdez* oil spill overlapped with the annual spring migration of herring spawners to near-shore staging areas (Norcross & Frandsen, 1996). Over 40% of the herring spawning, staging, and egg deposition areas and over 90% of the documented summer rearing and

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feeding areas were polluted with oil prior to spawning (Brown *et al.*, 1996). Damage to herring included instantaneous mortality as well as sublethal effects in newly hatched larvae including genetic damage (Brown *et al.*, 1996). In 1993, the total observed spawning population was less than one-third of preseason predictions and the average sizes of herring in each age class were some of the smallest on record. Aerial surveys in 1996 indicated that the herring population, while still depressed, had begun to recover (S. Grant, Alaska Department of Fish and Game, pers. comm.). If the forecast of an above-threshold biomass materializes in 1997, a commercial harvest will be permitted (due to the decline in numbers, no herring were harvested from 1993 to 1996).

Our goal was to contribute to the long-term conservation of Pacific herring in Prince William Sound by providing a basic understanding of the genetic relationships among aggregations of herring spawners both within the Sound and adjacent to the Sound in the Gulf of Alaska and Bering Sea. Allozyme electrophoresis has proved to be the most useful tool for delineating the population structure of many commercially important species in Alaska (e.g. Grant *et al.*, 1980; Gharret *et al.*, 1987; Wilmot *et al.*, 1994). Allozymes defined two distinct races of Pacific herring (Asian/Bering Sea and eastern North Pacific), with some evidence of further subdivision between the Gulf of Alaska and more southerly North Pacific stocks (Grant & Utter, 1984). However, previous surveys of herring species using this technique have generally revealed differentiation only over broad geographical regions (Grant & Utter, 1984) (although see Kobayashi *et al.*, 1990 and Jorstad *et al.*, 1994, for exceptions).

The application of microsatellites to resolve between closely related fish populations has increased rapidly over the last few years (e.g. McConnell *et al.*, 1995; Ruzzante *et al.*, 1996*a*; O'Connell *et al.*, 1997). Microsatellites generally consist of short (2–5 bp) variable tandemly repeated arrays which appear to be abundant and dispersed broadly throughout the eukaryote genome (Tautz & Renz, 1984). The very high levels of variation that are observed generally render this class of loci useful for population genetic analysis. Detection of variation at microsatellite loci employs the polymerase chain reaction (PCR) technique, and this feature has been primarily responsible for the increasing use of microsatellite markers in conservation genetics.

The objective of this paper is to describe the levels of genetic variation and divergence at five microsatellite loci for individuals collected from four sites within Prince William Sound, two sites within the Bering Sea and one site at Kodiak Island. The patterns of differentiation and variation are compared to those observed previously using allozymes.

MATERIALS AND METHODS

FIELD SAMPLING

During 1995, field collections of spawning Pacific herring targetted four representative sites within Prince William Sound. These sites were chosen to maximize the potential genetic differentiation among temporally and spatially isolated spawning aggregations. Tissue extracts from muscle, liver, eye, and heart were collected and preserved in liquid nitrogen for transport to -80° C freezers for storage until analysis.

The within-Sound sampling efforts in 1995 targetted Rocky Bay, a south-central spawning isolate on Montague Island; St Matthews Bay, a south-east isolate; Fish Bay,

TABLE I. Locations and abbreviations for samples of Pacific herring; location numbers as in Fig. 1

Location number	Sample size	Dates sampled	Latitude (N)	Longitude (W)	Location and timing	Location abbreviation
1	50	4/95	60°20′	146°20′	St Matthews Bay	PWS-SE
2	50	4/95	60°49′	146°25′	Fish Bay, early*	PWS-NE
3	50	4/95	60°21′	147°07′	Rocky Bay late*	PWS-RBL
4	50	4/95	60°15′	147°13′	Port Chalmers, late*	PWS-PCL
5	50	5/95	58°06′	153°04′	Kodiak Island	HKODE
6	50	5/91	58°50′	160°24′	Togiak Bay	HT
7	50	5/91	63°54′	160°50′	Norton Sound	HNS

*The absence of bimodal spawning activity has precluded within-year temporal sampling, early and late refer to timing of spawning relative to all of PWS within a collection year.

a north-east isolate, and Port Chalmers on Montague Island (Table I and Fig. 1). Efforts to sample both early- and late-spawning stocks within these four sites were unsuccessful in 1995 due to the timing of spawning returns and inclement weather. Single collections were made in these spawning sites. Early-spawning isolates were collected from St Matthews Bay and Fish Bay, and late-spawning isolates were collected from Rocky Bay and Port Chalmers. Additionally in 1995, samples were collected from an adjacent spawning population of Pacific herring from Kodiak Island for comparison with Prince William Sound populations. Samples from the geographically distant Bering Sea spawning populations in Togiak Bay and Norton Sound, which were collected in 1991, were also included.

DNA ANALYSIS

A detailed description of the primer development, DNA extraction protocols, PCR and running conditions are given elsewhere (O'Connell *et al.*, 1997*b*). Primer sequences have been submitted to GenBank and are available under the following accession numbers: *Cha*17 AF019993; *Cha*20 AF019989; *Cha*63 AF019987; *Cha*113 AF019994; *Cha*123 AF019992. For accurate scoring, all gels were run with three sets of size standards (M13 sequence) per gel. Furthermore, two samples were re-loaded three times across gels to increase the accuracy of scoring.

Genetic heterozygosity at each locus was calculated using MICROSAT (Goldstein *et al.*, 1995). Allele frequencies were calculated and tested against Hardy–Weinberg (H–W) proportions using Fisher's exact test (GENEPOP, Raymond & Rousset, 1995). Allele frequency heterogeneity among sites was estimated with the Markov–Chain chain approach using χ^2 probability values (Guo & Thompson, 1992) with GENEPOP. The probabilities that F values (Weir & Cockerham, 1984) associated with each locus and population were significantly different from zero were also calculated using GENEPOP.

FSTAT 1.2 (Goudet, 1996) was used to calculate θ and its associated confidence intervals (95% and 99%). The R_{ST} statistic and its associated confidence limits were also calculated to investigate the level of genetic structuring among populations using the RST-CALC program (Goodman, 1997). The confidence limits associated with the θ and R_{ST} coefficients were generated through bootstrapping over 1000 replications.

The genetic relationships between all pairs of populations were estimated using Reynold's *et al.* (1983) co-ancestry coefficient and the CONTML option in PHYLIP 3.5 (Felsenstein, 1989). Both methods generate a genetic distance coefficient that reflects allele frequency differences. The relationships among samples inferred from both methods were summarized with a neighbour-joining tree (Saitou & Nei, 1987) and a maximum likelihood tree, respectively. The recently derived $\delta\mu^2$ distance coefficient (Goldstein *et al.*, 1995) was also calculated and the relationship among populations

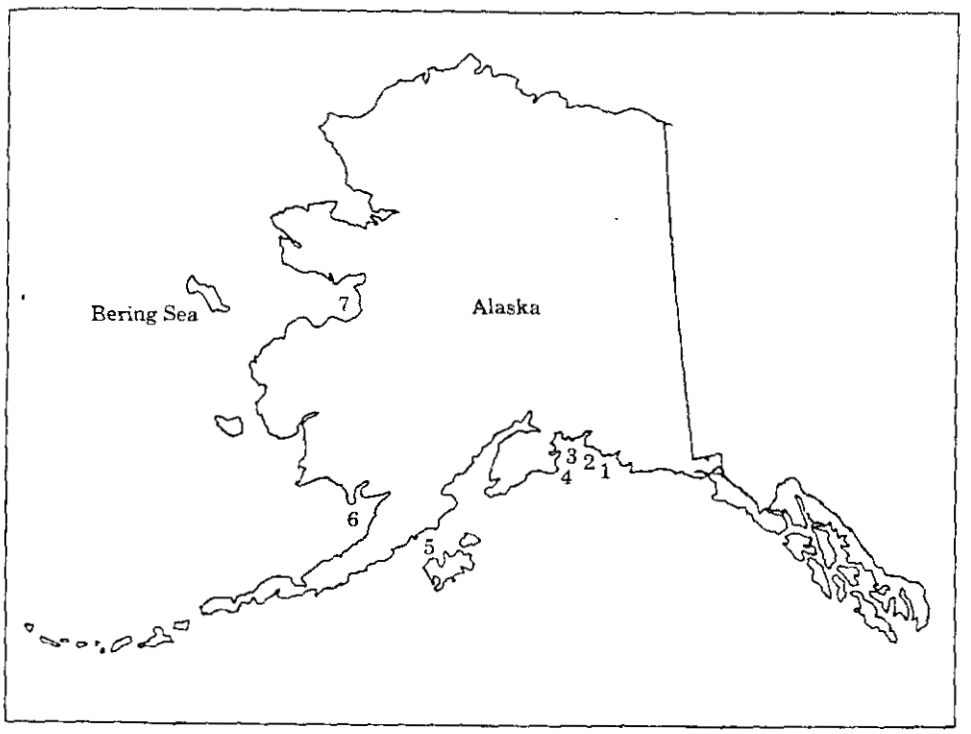

FIG. 1. Map showing the distribution of the sampling sites investigated. 1, PWS-SE district (St Matthews Bay); 2, PWS-NE district (Fish Bay); 3, PWS-Rocky Bay; 4, PWS-Pt Chalmers; 5, Kodiak West Side; 6, Togiak Bay; 7, Norton Sound.

demonstrated using neighbour-joining analysis. The bootstrap values for the trees were calculated over 1000 replications by bootstrap re-sampling across loci.

Individual alleles at each of the five loci were grouped into 10 or less separate size classes for the genetic analyses. The individual bin sizes were arranged so that rare alleles, commonly observed at the extremes of the allele frequency distribution range, were pooled in large bins. In contrast, the most common allele frequencies were grouped in much smaller bins to minimize frequency/sampling errors. The individual bin sizes (in base pairs) for each of the loci were: 98–120, 120–126, 128–132 and 134–142 for *Cha*20; 98–116, 118–126, 128–130, 132–134, 136–138, 140–142, 144–146, 148–160, 162–178 for *Cha*17; 128–144, 146–150, 152–156, 158–162, 164–168, 170–180 for *Cha*63; 100–114, 116–118, 120–122, 124–126, 128–132, 134–150 for *Cha*113 and finally, 150–164, 166–172, 174–180, 182–188, 190–196, 198–204, 206–230 for *Cha*123. For the R_{ST} analysis, each bin or allele grouping was designated to differ from its adjacent bin by a 2-bp increment.

RESULTS

POPULATION DIVERSITY AND DIVERGENCE

The average number of alleles per locus among samples was high ($k=33$) ranging from 46 for *Cha*123 to a low of 25 alleles for *Cha*113. There did not appear to be large differences among sites in the number of alleles. Also, heterozygosity estimates did not differ significantly between sites (t-test for arc sine transformed frequencies). Individual heterozygosity estimates ranged from 0·856 for *Cha*113 to 0·967 for *Cha*123 (Table II).

TABLE II. Range in allele sizes, number of alleles and heterozygosity estimates associated for each population at each locus

Location		Cha17	Cha20	Cha63	Cha113	Cha123
PWS-SE	Range in allele size	102–158	114–140	134–180	106–150	150–220
	No. of observed alleles	22	13	22	21	26
	Heterozygosity	0·911	0·890	0·917	0·892	0·939
PWS-NE	Range in allele size	102–182	112–136	130–180	104–144	158–218
	No. of observed alleles	24	12	21	19	22
	Heterozygosity	0·938	0·886	0·927	0·914	0·928
PWS-RBL	Range in allele size	102–166	112–144	130–180	106–146	160–226
	No. of observed alleles	26	15	20	21	22
	Heterozygosity	0·940	0·896	0·897	0·918	0·929
PWS-PCL	Range in allele size	102–178	100–138	130–168	100–138	162–218
	No. of observed alleles	21	15	19	16	26
	Heterozygosity	0·914	0·822	0·904	0·827	0·931
HKODE	Range in allele size	102–166	108–150	134–178	106–144	160–240
	No. of observed alleles	26	15	15	17	27
	Heterozygosity	0·917	0·897	0·883	0·856	0·942
HT	Range in allele size	98–146	98–138	128–178	100–140	124–228
	No. of observed alleles	20	14	18	15	26
	Heterozygosity	0·880	0·835	0·932	0·737	0·939
HNS	Range in allele size	100–152	112–142	130–170	108–140	158–230
	No. of observed alleles	19	14	20	13	30
	Heterozygosity	0·893	0·831	0·806	0·705	0·947

TABLE III. Probability values associated with the F (Weir & Cockerham, 1984) values for each locus

Site	Cha20	Cha17	Cha113	Cha123	Cha63
PWS-SE	0·023	+0·147	−0·086	+0·157	+0·001
PWS-NE	+0·151	−0·151	+0·076	+0·193	+0·009
PWS-RBL	+0·120	+0·010	−0·024	+0·411***	+0·078
PWS-PCL	+0·136	−0·018	−0·065	+0·365***	+0·291***
HKODE	+0·043	+0·057	+0·012	+0·276*	+0·142
HT	+0·058	+0·172	−0·031	+0·176	+0·035
HNS	+0·097	−0·115	−0·061	+0·258*	−0·017

*P<0·05; ***P<0·001.

An analysis of the binned data revealed all the sites investigated, excluding Point Chalmers and Rocky Bay, were in H–W proportions using Fisher's exact test. The significant result at both of these sites was due largely to a highly significant deficit of heterozygotes at *Cha*123. Large allele dropout and/or a high frequency of null alleles may be responsible for the significant deviation from H–W expectations at this locus. The probability values associated with the F (inbreeding) coefficients for each population and locus (excluding *Cha*123) revealed one significant value at *Cha*63 for the Point Chalmers site, after correcting for table-wide significance (Rice, 1989) (Table III). The significance was due to an excess of homozygotes.

The χ^2 tests on the binned data for total allele frequency heterogeneity identified highly significant allele frequency differences at all five loci among the sites investigated (P<0·001 for all loci). The θ value (0·036; S.D. 0·008) also revealed a highly significant level of genetic structuring among the populations investigated (P<0·001). The θ values calculated for each locus individually did not differ significantly from each other (95% confidence limits overlapped) and all were significantly different from zero (P<0·001). A comparison of pairwise θ values revealed that the populations from outside the Gulf of Alaska (Togiak Bay and Norton Sound) were approximately three times more distinct from populations within Prince William Sound than the Prince William Sound populations were from each other. The θ values also revealed the Kodiak Island and Port Chalmers samples to be relatively distinct from the remaining Prince William Sound samples (Table IV). The analogous R_{ST} coefficient, calculated through averaging the variance components over loci, also revealed a significant structuring of genetic variation among populations ($R_{ST(unbinned)}$=0·0542; P<0·001; $R_{ST(binned)}$=0·074; P<0·001). The R_{ST} coefficient confirmed the high degree of genetic isolation between populations from the Gulf of Alaska and the Bering Sea and also confirmed the relative distinctness of the Point Chalmers and Kodiak Island samples from the other Prince William Sound and Bering Sea samples.

GENETIC DISTANCE RELATIONSHIPS

Genetic relationships among samples, identified using three different distance coefficients were similar. The Togiak Bay and Norton Sound samples from the

TABLE IV. Pair-wise theta (θ) values between sample sites below the diagonal and pair-wise R_{ST} values above the diagonal

	PWS-SE	PWS-NE	PWS-RBL	PWS-PCL	HT	HNS	HKODE
PWS-SE	—	0·0066	0·0082	0·0543	0·1091	0·1640	0·2320
PWS-NE	0·0000	—	0·0000	0·0123	0·1091	0·1588	0·0045
PWS-RBL	0·0025	0·0000	—	0·0337	0·1103	0·1588	0·0113
PWS-PCL	0·0176	0·0126	0·0199	—	0·1097	0·1546	0·0027
HT	0·0516	0·0607	0·0794	0·0851	—	0·0084	0·0564
HNS	0·0620	0·0627	0·0783	0·0715	0·0054	—	0·0902
HKODE	0·0064	0·0108	0·0173	0·0075	0·0516	0·0529	—

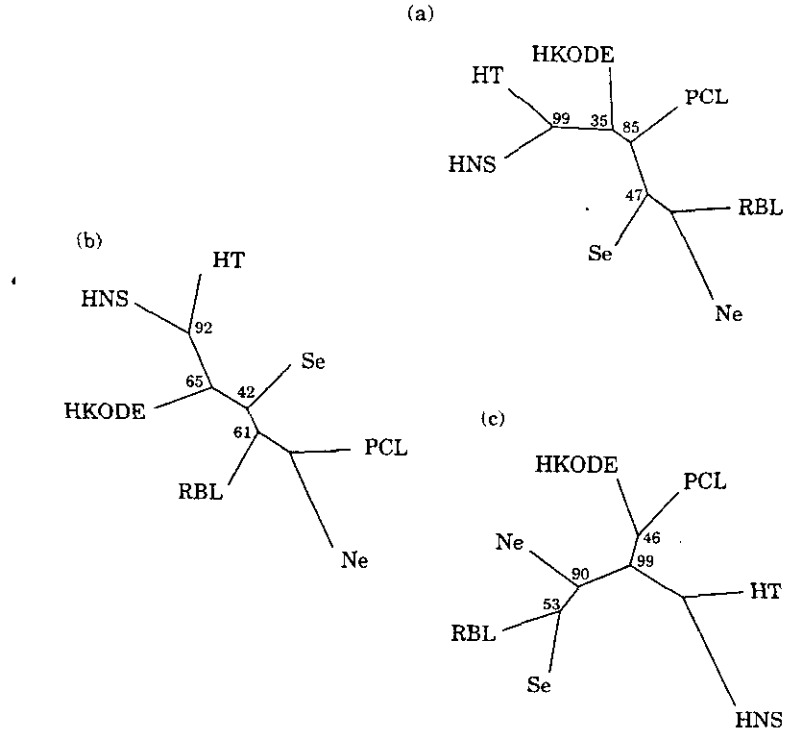

FIG. 2. (a) Neighbour-joining and maximum likelihood trees based on Reynolds *et al.* (1983) distance coefficient; (b) neighbour-joining tree based on $\delta\mu^2$ (Goldstein *et al.*, 1995); (c) maximum likelihood tree constructed using the CONTML option in PHYLIP (Felsenstein, 1989).

Bering Sea always clustered tightly together with a high degree of confidence (bootstrap value for this node was 96%). Distance estimates, derived from allele frequencies only, showed that the Kodiak Island and Port Chalmers sites were intermediate, situated between the Bering Sea and the remaining Prince William Sound samples. However, the tree derived from the $\delta\mu^2$ coefficient clustered the Port Chalmers site more closely to the remaining Prince William Sound samples than to the Kodiak Island sample. The Kodiak Island sample was again located between the Bering Sea and Prince William Sound population clusters as predicted from its geographic location (Fig. 2). One potential problem, which should be considered when interpreting the bootstrap values associated with genetic distance trees, is the artificially high robustness of nodes that can be generated through bootstrapping across a limited number of loci. The potential for artificially high values is due to the observation that the number of possible permutations may be considerably less than the number of re-sampling events (in this case 1000) and consequently lead to an artificially small variance estimate. Thus, re-sampling a large number of times with a small number of loci ($n=5$) can lead to a false interpretation of the robustness of the nodes identified. In the present case, the bootstrap values associated with the Bering Sea and Kodiak Island samples remained consistent when investigated using less replications (200). However, the values associated with the Prince William Sound populations did vary slightly.

DISCUSSION

WITHIN-POPULATION VARIATION

The Pacific herring populations surveyed in this study revealed very high levels of diversity with an average heterozygosity value of 0·889. The levels of diversity are similar to those reported for microsatellites in Atlantic cod *Gadus morhua* L. (Brooker *et al.*, 1994; Bentzen *et al.*, 1996; Ruzzante *et al.*, 1996*a*) and some anadromous fish species (O'Reilly *et al.*, 1996; Scribner *et al.*, 1996; O'Connell *et al.*, 1998). Similar high levels of diversity and range in allele size using the microsatellite loci developed for this study have also been observed with Atlantic herring *Clupea harengus* L. (P. Shaw, University of Hull, U.K., pers. comm.).

There did not appear to be any drastic reduction in the number of alleles or the heterozygosity estimates for any site that would be indicative of a recent bottleneck. Comparisons of heterozygosities and number of alleles between samples within Prince William Sound and all non-Prince William Sound samples also failed to reveal significant differences at this regional level. However, only very severe declines in population numbers would be evident from variation estimates derived from microsatellite data due to the very high levels of variation observed at this class of loci. Thus, it is not possible from the relative microsatellite variability estimates to infer that no reductions in population number have occurred as has been suggested by demographic data from Prince William Sound (Brown *et al.*, 1996). It should also be pointed out that the high number of alleles observed make any conclusion based on the number of private alleles or the presence/absence of alleles difficult. This is apparent when considering that on average only 61% of the potential number of alleles were described in the present study (where potential number of alleles per locus was defined as allele size range/2, i.e. length of repeat unit array). The very large numbers of alleles generally observed at microsatellite loci in coldwater fish species (O'Connell & Wright, 1997, and the references therein) increases the potential for sampling error in these species. It was this high potential for error that prompted the conservative but more statistically robust binning procedure.

A highly significant deficit of heterozygotes was observed for two populations at *Cha*123. Larger alleles at this locus often appeared feint and long exposures to film were generally necessary to score these alleles accurately. The significant deficit of heterozygotes is probably due to the failure of some larger alleles to amplify, i.e. null alleles (Callen *et al.*, 1993; Pemberton *et al.*, 1995). To account for the average deficit of heterozygotes at *Cha*123 (F=0·262), the null allele(s) would have an expected frequency of 0·131, which would mean that we would expect to observe one null homozygote for approximately every 50 individuals sampled. Comparisons of χ^2 tests for each sample revealed no significant difference between the number of samples failing to give a PCR product at this locus and the number of null homozygotes expected.

The significant probability value associated with the F coefficient for *Cha*63 was also due to a deficit of heterozygotes. Although null allele(s) are also considered the most likely explanation for these observations, the deficit of heterozygotes could also be due to demographic influences such as population admixture (Wahlund, 1928). If Wahlund's effect were responsible, the positive F values would be expected at a majority, if not all, of the loci at a particular site.

However, this was not observed to be the case at any of the sites investigated. Moreover, the level of differentiation between putative populations within Prince William Sound, the only site to show a significant deficit of heterozygotes at more than one locus, would have had to have been relatively high to generate the F values observed. Family effects could also explain the observed deviations from H–W expectations. Hedgecock (1994) proposed that sweepstakes recruitment might lead to a larger variance among families in reproductive success in marine animals. A high reproductive success for limited numbers of spawners is possible for many fish species due to their high gametic output. However, tests on fish species exhibiting similar life histories have shown no family effects (Ruzzante *et al.*, 1996*b*; Herbinger *et al.*, 1997). Furthermore, if sweepstake recruitment were responsible for the deviations from H–W expectations, such a mechanism would also be expected to generate significant deficits of heterozygotes over the majority of loci at a site, and this was not observed.

BETWEEN POPULATION DIVERGENCE

Significant allele frequency differences among populations were observed at all loci ($P<0{\cdot}001$). The pairwise values between sites also revealed significant differences in allele frequencies, after correcting for table-wide significance (Rice, 1989), which suggested that the samples investigated are largely reproductively isolated from each other. However, given the deviation from H–W proportions at two of the populations, the results from the χ^2 tests may be considered dubious as the alleles within individuals cannot be considered independent. Nevertheless, the bootstrap values associated with θ (0·036: 99%CL=0·020–0·052) and R_{ST} (0·074: 95%CL=0·064–0·108) also suggested that the collected samples do not represent a panmictic population. Furthermore, separate analyses of only those samples within the Gulf of Alaska also revealed a highly significant ($P<0{\cdot}001$) structuring of genetic variation for both coefficients. This was due predominately to the genetic distinctness of the Point Chalmers and Kodiak Island samples (note, a similar pattern and level of structuring was also observed for θ and R_{ST} using the unbinned data). In a direct comparison of the performance of the two statistics, the R_{ST} coefficient, which takes into account differences in allele size as well as frequency, consistently identified a higher level of differentiation. This finding was a little surprising given that a binning approach, which significantly reduced the range in allele sizes, was applied. From a fisheries perspective, the results do suggest some level of genetic structuring among samples. The pairwise θ and R_{ST} values (Table IV) showed very little or no evidence for genetic isolation among St Matthew's Bay, Fish Bay and Rocky Bay samples which were collected within Prince William Sound. However, the Port Chalmers sample did seem to be relatively distinct from the other Prince William Sound samples for both coefficients.

GENETIC DISTANCE VALUES

The genetic relationships among sites could be explained largely through the geographical distance between sampling locations. The two samples collected in the Bering Sea, Norton Sound and Togiak Bay, always clustered more closely with each other than with any other sample. The Kodiak Island sample was always intermediate in position between the Bering Sea and Prince William

Sound samples. However, it did vary slightly in terms of its relationship with Port Chalmers. The distance coefficients, which assume that genetic drift is the predominant structuring agent, clustered the Kodiak Island and Port Chalmers samples. Port Chalmers is geographically the closest of the Prince William Sound samples to Kodiak Island. However, the analysis based on the $\delta\mu^2$ distance coefficient showed no relationship between these two sites and the Port Chalmers sample was demonstrated to be much more similar to the other Prince William Sound samples.

The observed pattern of genetic relationships could have been caused by a temporal effect, i.e. temporal instability of allele frequencies, as the two Bering Sea populations were collected 4 years earlier than the other populations. However, Grant & Utter (1984) in an extensive survey of Pacific herring, which included collections from this area, observed no shifts in allele frequencies over time. It should also be noted that the observed high degree of divergence between populations from the Bering Sea and the Gulf of Alaska agree with a previous allozyme study of Pacific herring (Grant & Utter, 1984). This study identified a high level of genetic divergence between the Gulf of Alaska and Bering Sea and the authors proposed that herring from the two regions represented distinct genetic races. The origin of the two proposed races is probably related to isolation by Pleistocene coastal glaciation. Grant & Utter (1984) proposed that with the advance of the coastal glaciers, a physical barrier to gene flow was created between eastern and western Pacific populations of herring. With the retreat of the glaciers, herring from a Bering Sea refugium probably colonized shores and inlets along the Bering Sea. Pacific herring from a second more southern refugium (Pacific refugium) probably migrated north along the coast from California into the Gulf of Alaska. The more intermediate position of the Kodiak Island and Point Chalmers samples is consistent with the theory that the two proposed sources of colonization came into secondary contact around these areas and loci introgression is occurring. The results of an on-going mtDNA survey (P. Bentzen, unpubl. data) on these populations may provide more information on the phylogeography of the region.

In contrast to the allozyme study, the microsatellite analysis revealed some evidence for genetic structuring within the eastern Bering Sea. The relative distinctness of the Port Chalmers site from the other Prince William Sound samples contrasted with a previous allozyme survey that failed to find evidence of genetic differentiation within the Gulf of Alaska. The level of structuring between Kodiak Island and Port Chalmers, the easternmost of the Prince William Sound populations, was relatively low and this may reflect some level of straying between the two sites. The detection of relatively small-scale population units conforms to previous morphological and tagging data. Morphological data have demonstrated that numerous spawning grounds from California to south-eastern Alaska can be distinguished (Rounsefell & Dahlgren, 1935; cited in Grant & Utter, 1984). Furthermore, tagging data for herring along the British Columbia coast have revealed that for most management areas homing averaged 83·6% (Hourston, 1982). Although this figure would be expected to lead to a homogenization of allele frequencies, given the large effective population sizes of herring, the tagging data only demonstrate the presence of strays, only a fraction of which may represent genetically effective migrants.

In summary, the microsatellite markers revealed high levels of genetic diversity for the Pacific herring populations investigated. There was evidence to suggest genetic structuring within the Prince William Sound area but this conclusion will have to be verified with repeat sampling. In contrast, the much higher divergence estimates among the Bering Sea samples and samples within the Prince William Sound area indicated a high degree of genetic isolation in herring from these regions that generally confirms the findings of previous studies based on alternative data sets.

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References

Bentzen, P., Taggart, C. T., Ruzzante, D. E. & Cook, D. (1996). Microsatellite polymorphism and the population structure of Atlantic cod (*Gadus morhua*) in the Northwest Atlantic. *Canadian Journal of Fisheries and Aquatic Sciences* **53,** 2706–2721.

Brooker, A. L., Cook, D., Bentzen, P., Wright, J. M., & Doyle, R. W. (1994). Organization of microsatellites differs between mammals and cold-water teleost fishes. *Canadian Journal of Fisheries and Aquatic Sciences* **51,** 1959–1966.

Brown, E. D., Baker, T. T., Hose, J. E., Kocan, R. M., Marty, G. D., McGurk, M. D., Norcross, B. L. & Short, J. (1996). Injury to the early life history stages of Pacific herring in Prince William Sound after the *Exxon Valdez* Oil Spill. In *Proceedings of the Exxon Valdez Oil Spill Symposium, Bethesda, Maryland* (Rice, S. D., Spies, R. B., Wolfe, D. A. & Wright, B. A., eds). *American Fisheries Society Symposium* **18,** 448–462.

Callen, D. F., Thompson, A. D., Shen, Y., Phillips, H. A., Richards, R. I., Mulley, J. C. & Sutherland, G. R. (1993). Incidence and origin of null alleles in the $(AC)_n$ microsatellite markers. *American Journal of Human Genetics* **52,** 922–927.

Felsenstein, J. (1989). PHYLIP—phylogeny inference package (version 3.2). *Cladistics* **5,** 164–166.

Gharrett, A. J., Shirley, S. M. & Tromble, G. R. (1987). Genetic relationships among populations of Alaskan chinook salmon (*Oncorhynchus tshawytscha*). *Canadian Journal of Fisheries and Aquatic Sciences* **44,** 765–774.

Goldstein, D. B., Linares, A. R., Cavalli-Sforza, L. L. & Feldman, M. W. (1995). An evaluation of genetic distances for use with microsatellite loci. *Genetics* **139,** 463–471.

Goodman, S. J. (1997). RST CALC: A collection of computer programs for calculating unbiased estimates of genetic differentiation and gene flow from microsatellite data and determining their significance. *Molecular Ecology* **6,** 881–885.

Goudet, J. (1996). *F-STAT (1.2), A Program for IBM PC Compatible to Calculate Weir and Cockerham's (1984) Estimators of F-statistics.* Institut de Zoologie et d'Ecologie Animale, Bâtiment de Biologie, Université de Lausanne, CH-1015 Dorigny, Switzerland.

Grant, W. S. & Utter, F. M. (1984). Biochemical population genetics of Pacific herring (*Clupea pallasi*). *Canadian Journal of Fisheries and Aquatic Sciences* **41,** 856–864.

Grant, W. S., Milner, G. B., Krasnowski, P. & Utter, F. M. (1980). Use of biochemical genetic variants for identification of sockeye salmon (*Oncorhynchus nerka*) stocks in Cook Inlet, Alaska. *Canadian Journal of Fisheries and Aquatic Sciences* **37,** 1236–1247.

Guo, S. W. & Thompson, E. A. (1992). Performing the exact test of Hardy–Weinberg proportion for multiple alleles. *Biometrics* **48**, 361–372.

Hedgecock, D. (1994). Does variance in reproductive success limit effective population sizes of marine organisms? In *Genetics and Evolution of Aquatic Organisms* (Beaumont, A. R., ed.), pp. 122–134. London: Chapman and Hall.

Herbinger, C. M., Doyle, R. W., Taggart, C. T., Lochmann, S. E., Brooker, A. L., Wright, J. M. & Cook, D. (1997). Family relationships and effective population size in a natural cohort of cod larvae. *Canadian Journal of Fisheries and Aquatic Sciences* **54**, 11–18.

Hourston, A. S. (1982). Homing by Canada's west coast herring to management units and divisions as indicated by tagging recoveries. *Canadian Journal of Fisheries and Aquatic Sciences* **39**, 1414–1422.

Jorstad, K. E., Dahle, G. & Paulsen, O. I. (1994). Genetic comparison between Pacific herring *Clupea pallasi*) and a Norwegian fjord stock of Atlantic herring (*Clupea harengus*). *Canadian Journal of Fisheries and Aquatic Sciences* **51** (Suppl.1), 233–239.

Kobayashi, T., Iwata, M. & Numachi, K. (1990). Genetic divergence among local spawning populations of Pacific herring in the vicinity of northern Japan. *Bulletin of the Japanese Society of Science and Fisheries* **56**, 1045–1052.

McConnell, S. K. J., O'Reilly, P., Hamilton, L., Wright, J. M. & Bentzen, P. (1995). Polymorphic microsatellite loci from Atlantic salmon (*Salmo salar*): Genetic differentiation of North American and European populations. *Canadian Journal of Fisheries and Aquatic Sciences* **52**, 1863–1872.

Norcross, B. L. & Frandsen, M. (1996). Distribution and abundance of larval fishes in Prince William Sound, Alaska during, 1989 after the *Exxon Valdez* Oil Spill. In *Proceedings of the Exxon Valdez Oil Spill Symposium, Bethesda, Maryland* (Rice S. D., Spies, R. B., Wolfe, D. A. & Wright, B. A., eds). *American Fisheries Society Symposium* **18**, 463–486.

O'Connell, M. & Wright, J. M. (1997). Microsatellite DNA in fishes. *Reviews in Fish Biology and Fisheries* **7**, 331–363.

O'Connell, M., Danzmann, R. G., Cornuet, J.-M., Wright, J. M. & Ferguson, M. M. (1997). Differentiation of rainbow trout populations in Lake Ontario and the evaluation of the stepwise mutation and infinite allele mutation models using microsatellite variability. *Canadian Journal of Fisheries and Aquatic Sciences* **54**, 1391–1399.

O'Connell, M., Dillon, M. C. & Wright, J. M. (1998). Development of primers for polymorphic microsatellite loci in the Pacific herring (*Clupea harengus pallasi*). *Molecular Ecology*, in press.

O'Reilly, P., Hamilton, L. C., McConnell, S. K. & Wright, J. M. (1996). Rapid analysis of genetic variation in Atlantic salmon (*Salmo salar*) by PCR multiplexing of dinucleotide and tetranucleotide microsatellites. *Canadian Journal of Fisheries and Aquatic Sciences* **53**, 2292–2298.

Pemberton, J. M., Slate, J., Bancroft, D. R. & Barret, J. A. (1995). Non-amplifying alleles at microsatellite loci: a caution for parentage and population studies. *Molecular Ecology* **4**, 249–252.

Raymond, M. & Rousset, F. (1995). GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. *Journal of Heredity* **86**, 248–249.

Reynolds, J., Weir, B. S. & Cockerham, C. C. (1983). Estimation of co-ancestry coefficient: basis for a short-term genetic distance. *Genetics* **105**, 767–779.

Rice, W. R. (1989). Analyzing tables of statistical tests. *Evolution* **41**, 223–225.

Rousefell, G. A. & Dahlgren, E. H. (1935). Races of herring, *Clupea pallasi*, in southeastern Alaska. *Bulletin of United States Bureau of Fish* **48**, 119–141.

Rowell, K. A., Geiger, H. J. & Bue, B. G. (1990). Stock identification of Pacific herring in the Eastern Bering Sea trawl bycatch and in the Dutch Harbor directed food and bait fishery. In *Proceedings of the International Herring Symposium, Alaska Sea Grant Program*, Report No. 91-01, pp. 255–278. Fairbanks: University of Alaska.

Ruzzante, D. E., Taggart, C. T., Cook, D. & Goddard, S. (1996*a*). Genetic differentiation between inshore and offshore Atlantic cod (*Gadus morhua* L.) off Newfoundland: microsatellite DNA variation and anti-freeze level. *Canadian Journal of Fisheries and Aquatic Sciences* **53**, 634–645.

Ruzzante, D. E., Taggart, C. T. & Cook, D. (1996*b*). Spatial and temporal variation in the genetic composition of a larval cod (*Gadus morhua*) aggregation: cohort contribution and genetic stability. *Canadian Journal of Fisheries and Aquatic Sciences* **53**, 2695–2705.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* **4**, 406–425.

Schweigert, J. F. (1993). Evaluation of harvesting policies for the management of Pacific herring stocks, *Clupea pallasi*, in British Columbia. In *Management Strategies for Exploited Fish Populations* (Kruse, G., Eggers, M., Marasco, R. J., Pautzke, C. & Quinn, T. R., eds), pp. 167–190. Fairbanks: Alaska Sea Grant College Program, University of Alaska, Report No. 93-02.

Schweigert, J. F. & Withler, R. E. (1990). Genetic differentiation of Pacific herring based on enzyme electrophoresis and mitochondrial DNA analysis. *American Fisheries Society Symposium* **7**, 459–469.

Scribner, K. T., Gust, J. R. & Fields, R. L. (1996). Isolation and characterization of novel salmon microsatellite loci: cross-species amplification and population genetics applications. *Canadian Journal of Fisheries and Aquatic Sciences* **53**, 833–841.

Tautz, D. & Renz, M. (1984). Simple sequences are ubiquitous repetitive components of eukaryotic genomes. *Nucleic Acids Research* **12**, 4127–4138.

Wahlund, S. (1928). The combination of population and the appearance of correlation examined from the stand-point of the study of heredity. *Hereditas* **11**, 65–106 (in German).

Weir, B. S. & Cockerham, C. C. (1984). Estimating F-statistics for the analysis of population structure. *Evolution* **38**, 1358–1370.

Wilmot, R. L., Everett, R. J., Spearman, W. J., Baccus, R., Varnavskaya, N. V. & Putivkin, S. V. (1994). Genetic stock structure of Western Alaska chum salmon and a comparison with Russian far East stocks. *Canadian Journal of Fisheries and Aquatic Sciences* **51** (Suppl. 1), 95–113.

APPENDIX C

FINAL REPORT: TEMPORAL STABILITY OF MICROSATELLITE MARKERS IN PRINCE WILLIAM SOUND HERRING POPULATIONS

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FINAL REPORT: TEMPORAL STABILITY OF MICROSATELLITE MARKERS IN PRINCE WILLIAM SOUND HERRING POPULATIONS

Introduction

The work presented here follows that reported in "Development of microsatellite markers for genetic discrimination of Prince William Sound herring populations", submitted by Jonathan Wright, Michael O'Connell and Mary Dillon to Alaska Department of Fish and Game in September, 1996. The primary objective of the current research was to assess temporal stability in the microsatellite markers developed for Pacific herring. However, this report will first give details of the 1996 herring samples analysed alone, to determine whether the same patterns observed in 1995 were also seen in 1996. The data for the 1995-96 comparison will then be presented. We found the results from the 1996 samples analysed alone much like those of the 1995 samples, in that allele frequencies were significantly different in different populations, and that populations outside the Gulf of Alaska were more distant from populations within the Gulf than those within were from each other. The temporal analysis showed that in general, 1995 populations clustered together as did the 1996 ones.

Methods

Laboratory methods

DNA was extracted from muscle using proteinase K/SDS as described in O'Connell et al. (submitted). Extractions were done for 50 individual from each of 7 populations. Primer labeling and PCR was carried out as described in O'Connell et al. (submitted), except that P-33 was used for some of the assays. Populations were assayed at five microsatellite loci: Cha 17, Cha 20, Cha 63, Cha 113 and Cha 123 (O'Connell et al., in press).

PCR products were loaded on 8% denaturing gels and run at 60-70 mA for 2.5 to 4.5 hours, depending on the size of the locus. Each gel contained 3 sets of size markers (M13 sequence). In addition, to facilitate comparisons between years and between gels, allelic size standards were run 6 times on every gel (*i.e.* every 8 lanes). These allelic standards consisted of PCR products from the 1995 samples. Four 1995 samples for each locus were selected to span the expected range of allele sizes, and to include common allele sizes. The PCR products for the standards were combined and run in one lane, repeated across gels.

Analysis methods

Analysis was carried out essentially as described in O'Connell (submitted) . Genetic heterozygosity at each locus was calculated using MICROSAT (Goldstein et al., 1995). GENEPOP (v.1.2) (Raymond and Rousset, 1995) was used to test allele frequencies against Hardy-Weinberg (H-W) predictions, estimate allele frequency heterogeneity, and calculate χ^2 values associated with $f(F_{is})$. θ (F_{st}) and its associated confidence intervals (95% and 99%) were calculated using FSTAT 1.2 (Goudet, 1996). The estimated number of migrants was calculated using the private allele model (Barton and Slatkin, 1984). The genetic relationships between all pairs of populations were estimated using Cavalli-Sforza and Edward's (1967), Reynold's et al. (1983) co-ancestry coefficient and the CONTML option in PHYLIP 3.5 (Felsenstein, 1989). The relationships derived from these methods were summarized using neighbor-joining trees (Saitou and Nei, 1987) and a maximum likelihood tree. Relationships among the populations were also calculated using Goldstein et al.'s (1995) distance coefficient ($\delta\mu^2$), using the program MICROSAT. Bootstrap values for the trees were calculated by resampling across all loci 100 times.

Results

1996 data

In the 1996 samples, the average number of alleles per locus was 34, with a range of 23 for Cha 113 to 50 for Cha 123 (Table 1). Average heterozygosity for the 5 loci was 0.903.

F_{is} values for each locus and associated probability values are shown in Table 2. Thirteen non-random values were observed.

χ^2 tests revealed significant allele frequency heterogeneity among the sites investigated, with probability values $p < 0.001$ for all loci. The overall θ value (0.018, Std. Dev. 0.002) revealed highly significant genetic structuring among the populations ($p<0.001$ for all loci). Pair-wise θ values revealed that the populations outside the Gulf of Alaska (Togiak Bay and Norton Sound) were more distant from those within the Gulf, than the latter were from each other (Table 3).

Genetic distance relationships

Four distance coefficients were used to infer genetic relationships among populations (Figure 1 a-d). Although some patterns were clear regardless of distance method used, there was no overall consensus of tree topology. The Togiak Bay and Norton Sound populations always clustered together, with bootstrap values of 89 to 100%. The relationships amongst the remaining populations varied somewhat, depending on analysis method. For example, while the Kodiak Island population is intermediate between the Togiak Bay and Norton Sound populations in one analysis (Fig. 1c), this is not always the case.

Temporal Stability

Allele frequencies for each population sampled over two years are shown in Figures 2 - 6.

Distance relationships derived from four different analysis methods are shown in Figures 7 a-d. As for the 1996 data alone, some trends are obvious, but exact topology varies with distance measure. In all cases, the Togiak Bay and Norton Sound populations for both years group away from the rest of the samples. In addition, among these samples outside the Gulf of Alaska, the populations group more closely along years (HT 1996 with HNS 1996) as opposed to the same population clustering more closely over the two sampling years.

For the samples within the Gulf of Alaska, it is clear that populations sampled over two periods do not cluster together, and in general 1995 samples group together, as do 1996 samples. In some cases samples from two year classes group most closely, but often these nodes are poorly-supported by bootstrap values (*e.g.* Fig 7 d: Se95 and Ne 96 cluster, but only in 21% of cases).

Table 4 shows the results of χ^2 analyses for each population at each locus over the two sampling years. These results are presented with a note of caution, however, as some of the assumptions of the χ^2 analysis are invalidated if the populations are not in H-W equilibrium. However, this analysis shows that all populations changed allele frequency significantly at one or more loci.

Discussion

1996 data

For all loci except Cha 113, more alleles were seen in the 1996 assays than in 1995 (Table 1). For Cha 17, this could be attributed to the larger sample size obtained this year, thereby providing more opportunity to see additional alleles. For the other loci, however, sample sizes between the years were similar. It is possible that assay conditions contributed to the greater number of alleles observed in 1996. The use of the isotope P-33 in place of P-32 for some of the assays provided greater resolution, such that alleles were much clearer and sharper, and therefore more easily distinguished. In addition, we used knowledge gained in the 1995 assays to maximize the number of alleles observed; for Cha 123, all gels were exposed for at least four days, because we observed that some of the larger alleles at this locus were very faint in 1995.

As in 1995, significant allele frequency heterogeneity among populations was observed at all loci ($p<0.001$), indicating that the samples analysed were not from a panmictic population.

Pairwise θ values (Table 3) show the greater genetic distance between populations from outside the Gulf of Alaska than among those within the Gulf. Thus, the estimated number of genetic migrants to these outlying populations (Togiak Bay and Norton Sound) is low (between six and nine individuals), compared to the estimates within the Gulf (Table 3). The most surprising result from these estimates is the high number of migrants between the Kodiak Island population, where estimates range from 80 individuals between the North East district of Prince William Sound (PWS) and Kodiak Island, to 256 between Rocky Bay and this population. These estimates are much higher than the 1995 ones, where only six to seven individuals were estimated to migrate between these populations.

The genetic distance relationships inferred depended on the analysis method used. One common feature was the tight clustering of the populations outside the Gulf of Alaska (Figure 1). The exact topology of the remaining populations differed with different methods. In the 1995 analysis, the Kodiak Island and Pt. Chalmers populations usually (but not always) fell in between the remaining PWS samples and those outside the Gulf of Alaska, such that genetic distance correlated well with geographic distance. In 1996, this is not the case. The position of the Pt. Chalmers and Kodiak Island samples changes with analysis method. For example, the Kodiak Island population is most distant from the PWS ones in only one of the analyses (Fig. 1 c). For the other analyses the position of Kodiak Island relative to the PWS populations varies, but it is worth noting that bootstrap values on most nodes (with the exception of those for the Togiak and Norton Sound populations) are low, and should be viewed cautiously.

Temporal Stability

The frequency distributions presented in Figures 2 - 6 do not reveal any obvious, overall trends. For some populations and some loci, the distributions are rather similar over the sampling periods (*e.g.* Cha 113, HNS population). For others, however, the frequencies are quite different (*e.g.* Cha 20, PCL population). It also appears that some loci were more different across year classes than others. There was a highly significant difference at five out of seven populations at Cha 63, whereas only three populations appeared significantly different at Cha 123. These results could reflect real, biological trends, or could be related to the characteristics of the particular loci. Every effort was made to increase scoring consistency across years: the same person scored all 1995 and 1996 gels, and all 1996 gels

contained size standards from 1995. However, scoring is always difficult and must at least be considered as a potential source of error.

The genetic distance relationships inferred from four different measures (Fig. 7 a - d) clearly distinguish between samples within the Gulf of Alaska and those outside this area. The relationships of the populations within the Gulf are not clear, and many nodes are poorly-supported. It does seem, however, that clustering was among years rather than populations from two years clustering together (e.g. Fig. 7c, Se 96, Ne 96 PCL 96, and RBL 96 group together.

References

Barton, N. and Slatkin, M. (1986) A quasi-equilibrium theory of the distribution of rare alleles in a sub-divided population. *Heredity* 56: 409-415.

Cavalli-Sforza, L. L., and A. W. F. Edwards. 1967. Phylogenetic analysis: models and estimation procedures. Evolution 32: 550-570..

Felsenstein, J. 1989. PHYLIP -- Phylogeny Inference Package (Version 3.2). Cladistics 5: 164-166.

Goldstein, D. B., Linares, A.R., Cavalli-Sforza, L.L. and Feldman, M.W. (1995) An evaluation of genetic distances for use with microsatellite loci. Genetics 139: 463-471.

Goudet, J. 1996. F-STAT (1.2), a program for IBM PC compatible to calculate Weir and Cockerham's (1984) estimators of F-statistics.

O'Connell, M., Dillon, M.C. and Wright, J.M. Development of primers for polymorphic microsatellite loci in the Pacific herring (*Clupea harengus pallasi*). Molecular Ecology, in press.

O'Connell, M., Dillon, M.C., Wright, J.M., Bentzen, P., Merkouris, S. and Seeb, J. Genetic structuring among Alaskan Pacific herring (*Clupea pallasi*) populations identified using microsatellite variability. Submitted to Canadian Journal of Fisheries and Aquatic Sciences.

Raymond, M. and Rousset, F. (1995) GENEPOP Version 2.0. Laboratoire de Génétique et Environment, URA CNRS 327, Institut des Sciences de l'Evolution, CC 065, USTL.

Reynolds, J., Weir, B.S. and Cockerham, C.C. 1983. Estimation of co-ancestry coefficient: Basis for a short-term genetic distance. Genetics 105: 767-779

Saitou, N. and Nei, M. (1987) The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4: 406-425.

Table 1: Range of allele sizes, numbers of alleles, heterozygosity and sample sizes for each locus in 1995 and 1996

	# alleles		size range		heterozygosity		sample size	
	1995	1996	1995	1996	1995	1996	1995	1996
Cha 17	36	40	98-182	92-182	0.913	0.920	334	348
Cha 20	21	26	98-150	98-178	0.865	0.889	344	342
Cha 63	26	31	128-180	128-198	0.911	0.907	348	340
Cha 113	24	23	100-150	102-150	0.836	0.860	346	346
Cha 123	42	50	150-240	140-248	0.937	0.903	340	340
AVERAGE	30	34			0.892	0.903	342	343

Table 2: Probability values associated with the F_{is} values for each locus.

Site	Cha 17	Cha 20	Cha 63	Cha 113	Cha 123
Se	+.083	+.114	+.073*[1]	+.128	+.209
Ne	+.040	-.032	+.056	-.112	+.013
RBL	+.0231***	+.164**	-.009	+.034	+.154***
PCL	+.168***	+.169***	+.119	-.051*	+.113*
HT	-.091	+.080	+.146	+.059***	+.045
HNS	+.106*	+.039	+.089	+.108	+.082***
HKODE	+.033***	+.046	-.031	-.046	+.092*

1. $p < 0.05$ = *; $p < 0.01$ = **; $p < 0.001$ = ***

Se - PWS-Se district: St. Matthew's Bay
Ne - PWS-Ne district: Fish Bay
RBL - PWS-RBL district: Rocky Bay
PCL - PWS-PCL district: Point Chalmers
HT - Togiak Bay
HNS - Norton Sound
HKODE - Kodiak, west side

Table 3: Pair-wise theta (θ) values between sample sites below the diagonal, and the estimated number of genetic migrants above the diagonal

	Se	Ne	RBL	PCL	HT	HNS	HKODE
Se	---	43	95	41	8	6	128
Ne	0.0057	---	347	47	7	6	80
RBL	0.0026	0.0007	---	41	9	7	256
PCL	0.0061	0.0052	0.0061	---	8	7	33
HT	0.0292	0.0333	0.0281	0.0311	---	55	9
HNS	0.0374	0.0406	0.0364	0.0363	0.0045	---	7
HKODE	0.0020	0.0031	0.0010	0.0075	0.0263	0.0343	---

Se - PWS-Se district: St. Matthew's Bay
Ne - PWS-Ne district: Fish Bay
RBL - PWS-RBL district: Rocky Bay
PCL - PWS-PCL district: Point Chalmers
HT - Togiak Bay
HNS - Norton Sound
HKODE - Kodiak, west side

Table 4: χ^2 tests of populations assayed over two sampling periods.

	Cha 17	Cha 20	Cha 63	Cha 113	Cha 123
Se 95 & 96	*	n.s.	n.s.	***	n.s.
Ne 95 & 96	*	***	***	*	***
RBL 95 & 96	*	n.s.	n.s.	n.s.	n.s.
PCL 95 & 96	**	***	***	***	n.s.
HT 91 & 96	**	***	***	***	***
HNS 91 & 96	n.s.	**	***	n.s.	**
HKODE 95 & 96	n.s.	*	***	n.s.	n.s.

$p < 0.05$ = *
$p < 0.01$ = **
$p < 0.001$ = ***
n.s. = not significant

Se - PWS-Se district: St. Matthew's Bay
Ne - PWS-Ne district: Fish Bay
RBL - PWS-RBL district: Rocky Bay
PCL - PWS-PCL district: Point Chalmers
HT - Togiak Bay
HNS - Norton Sound
HKODE - Kodiak, west side

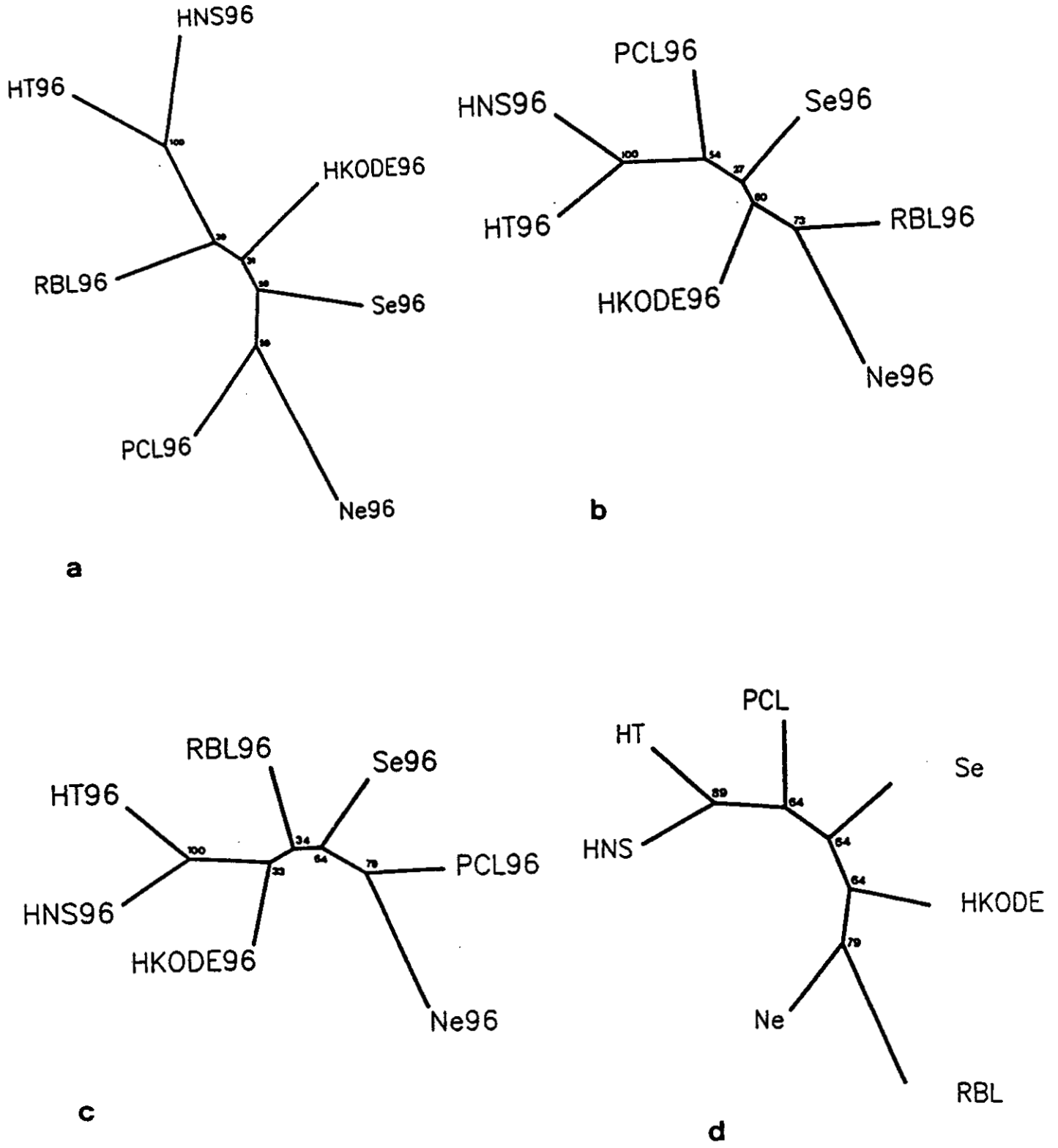


Figure 1. Genetic distance relationships for 1996 populations based on a) Cavalli-Sfortza distance; b) Reynold's distance; c) CONTML; and d) $\delta\mu^2$. Values on each node represent bootstrap values from 100 replicates.

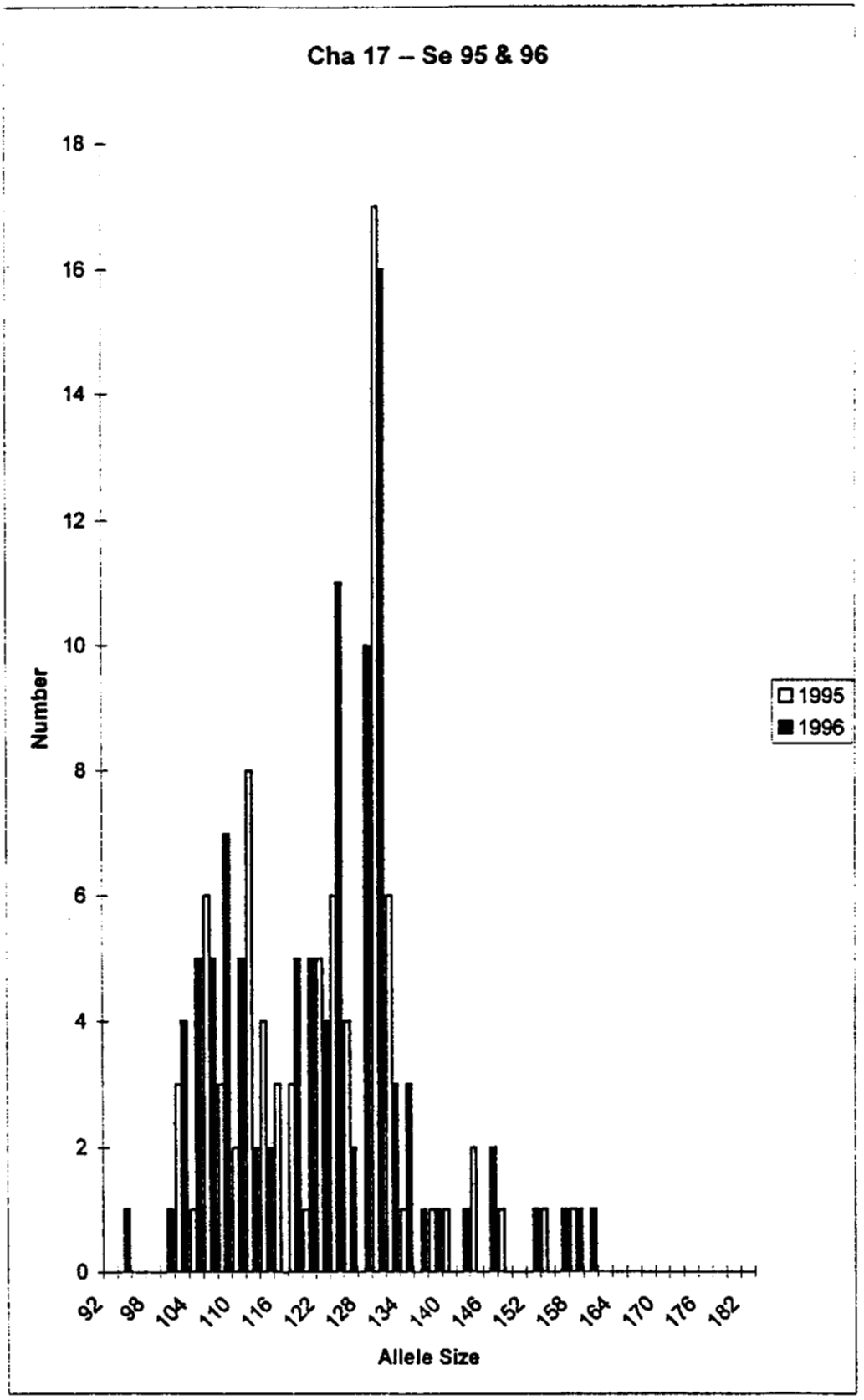


Figure 2. Allele frequencies of Cha 17 for each population sampled over two years.

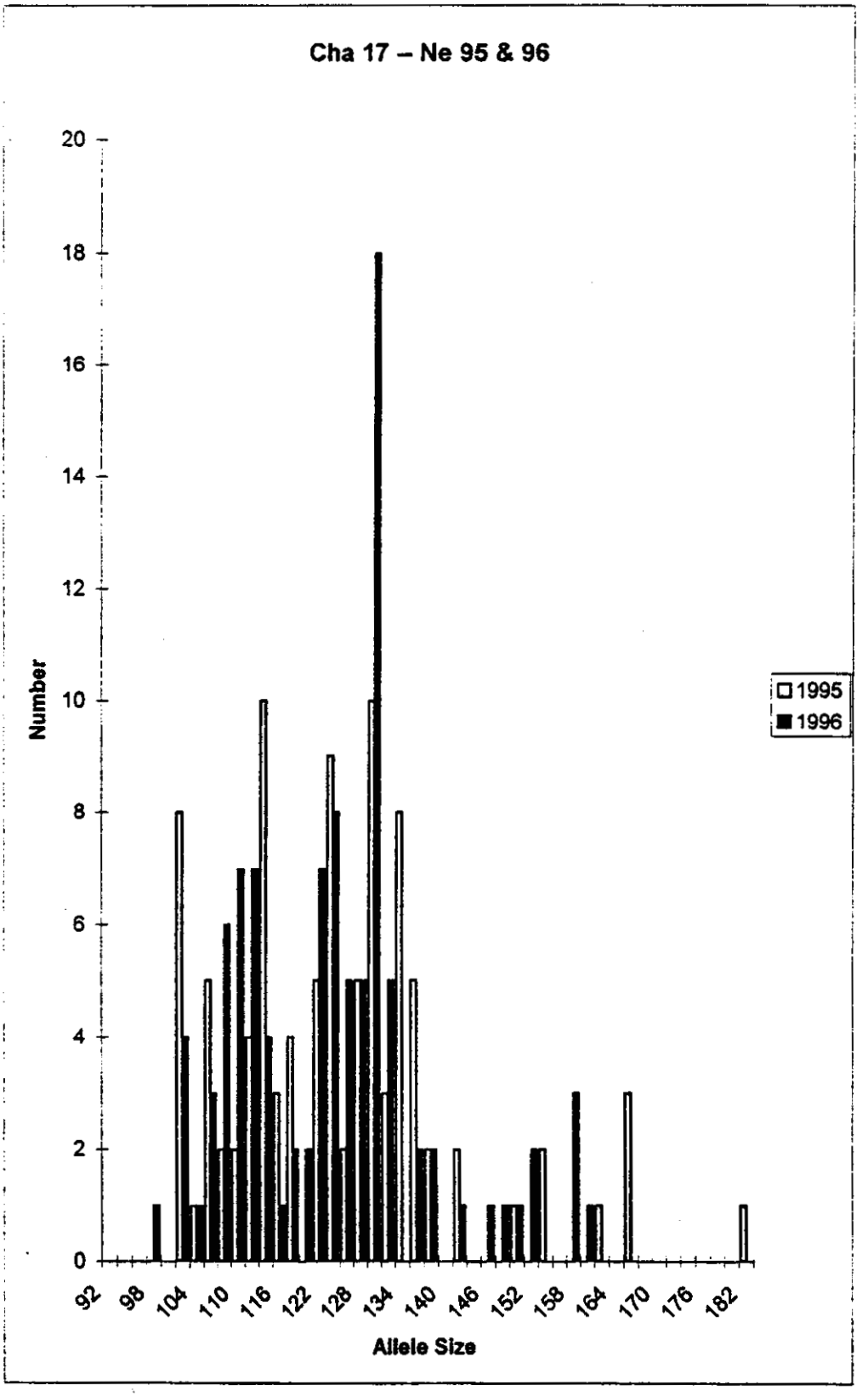
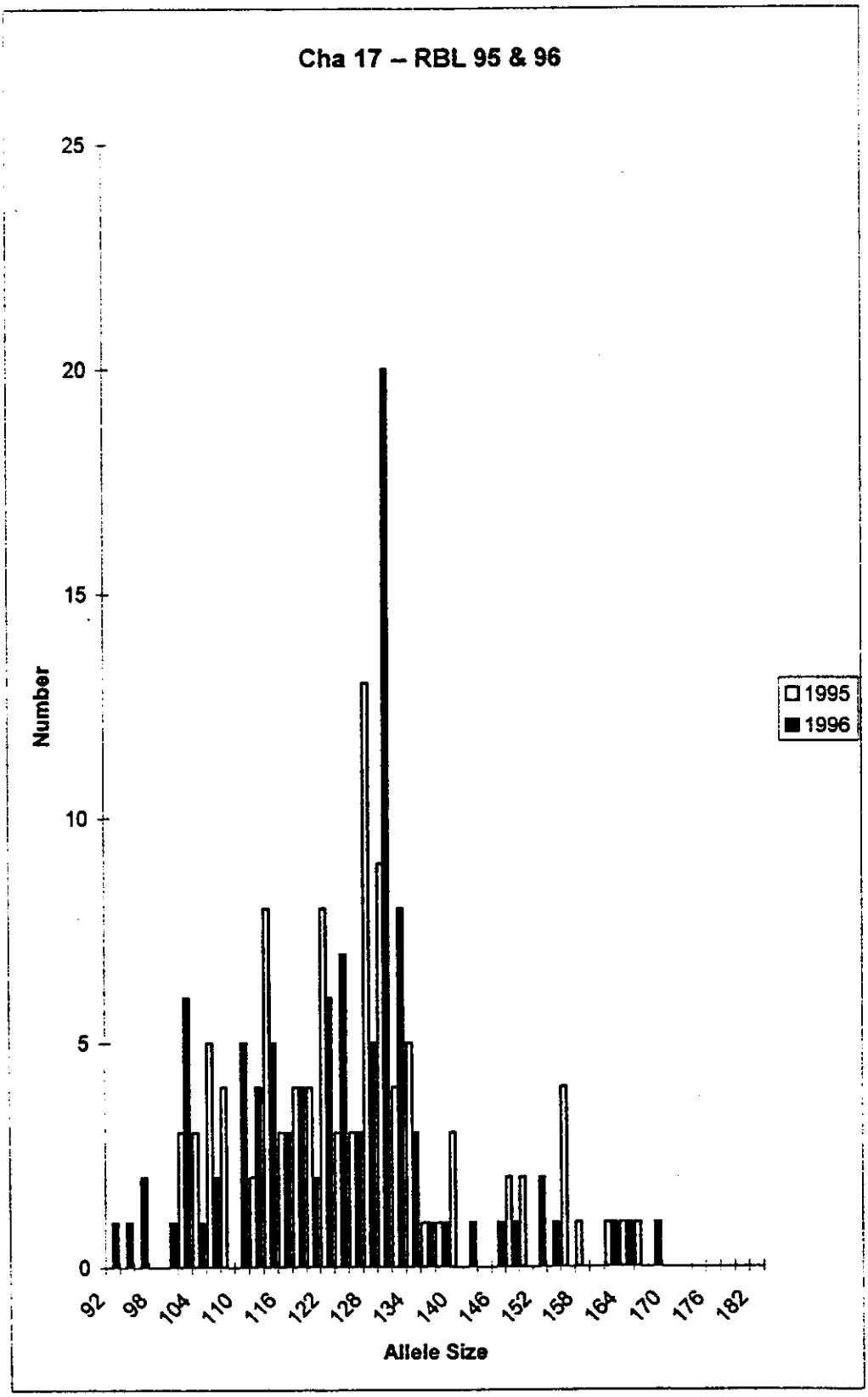


Figure 2. Allele frequencies of Cha 17 for each population sampled over two years.



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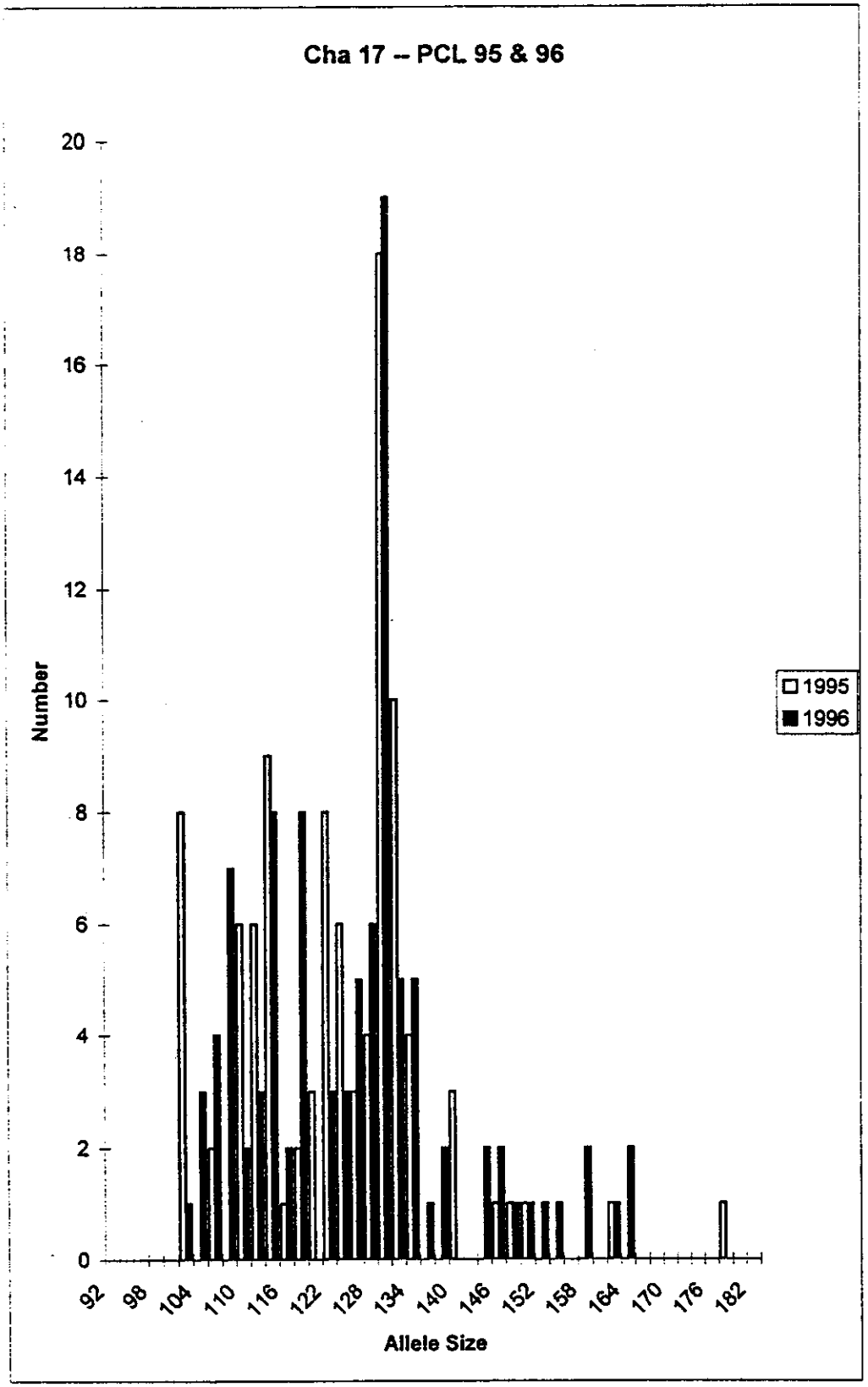


Figure 2. Allele frequencies of Cha 17 for each population sampled over two years.

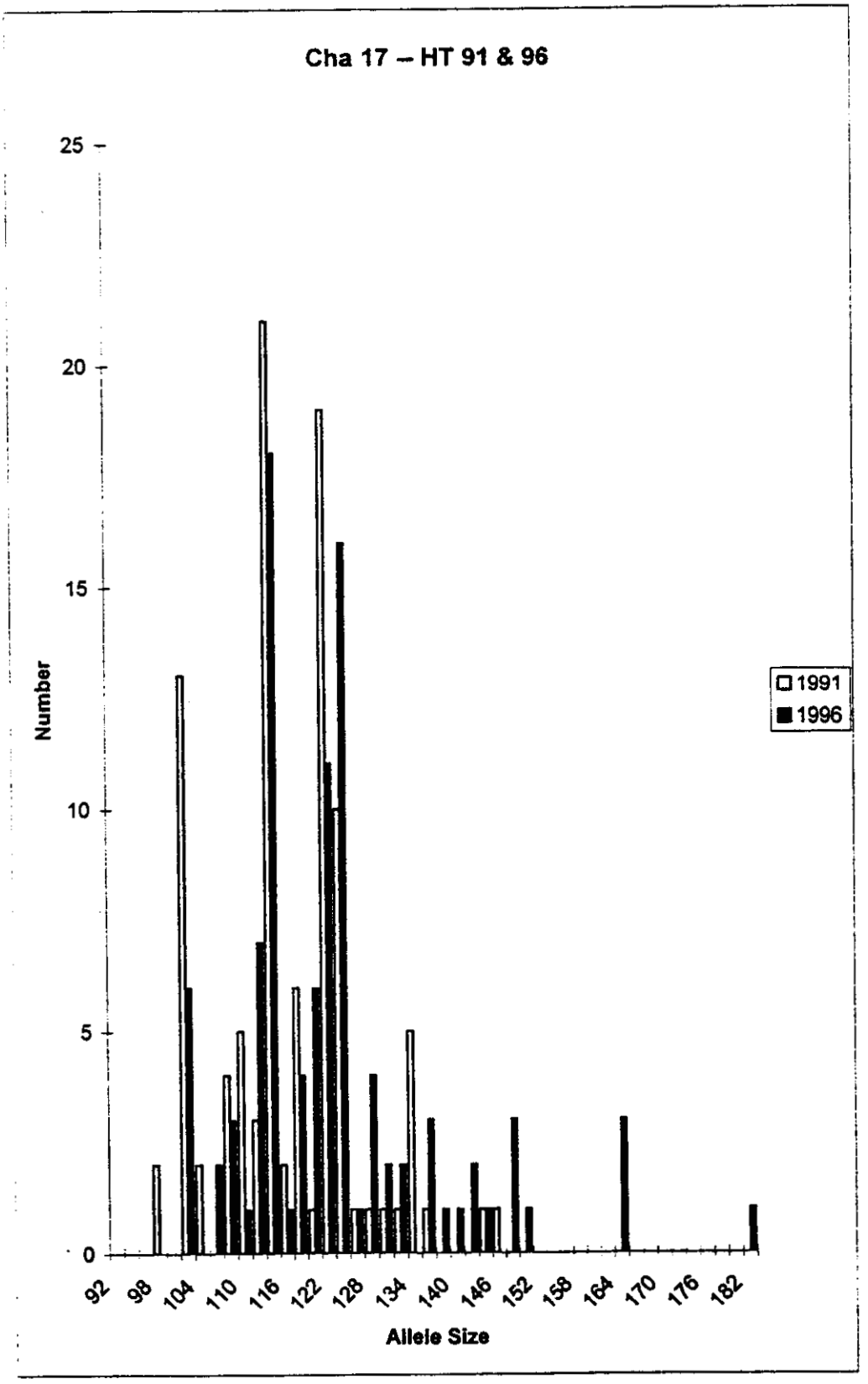
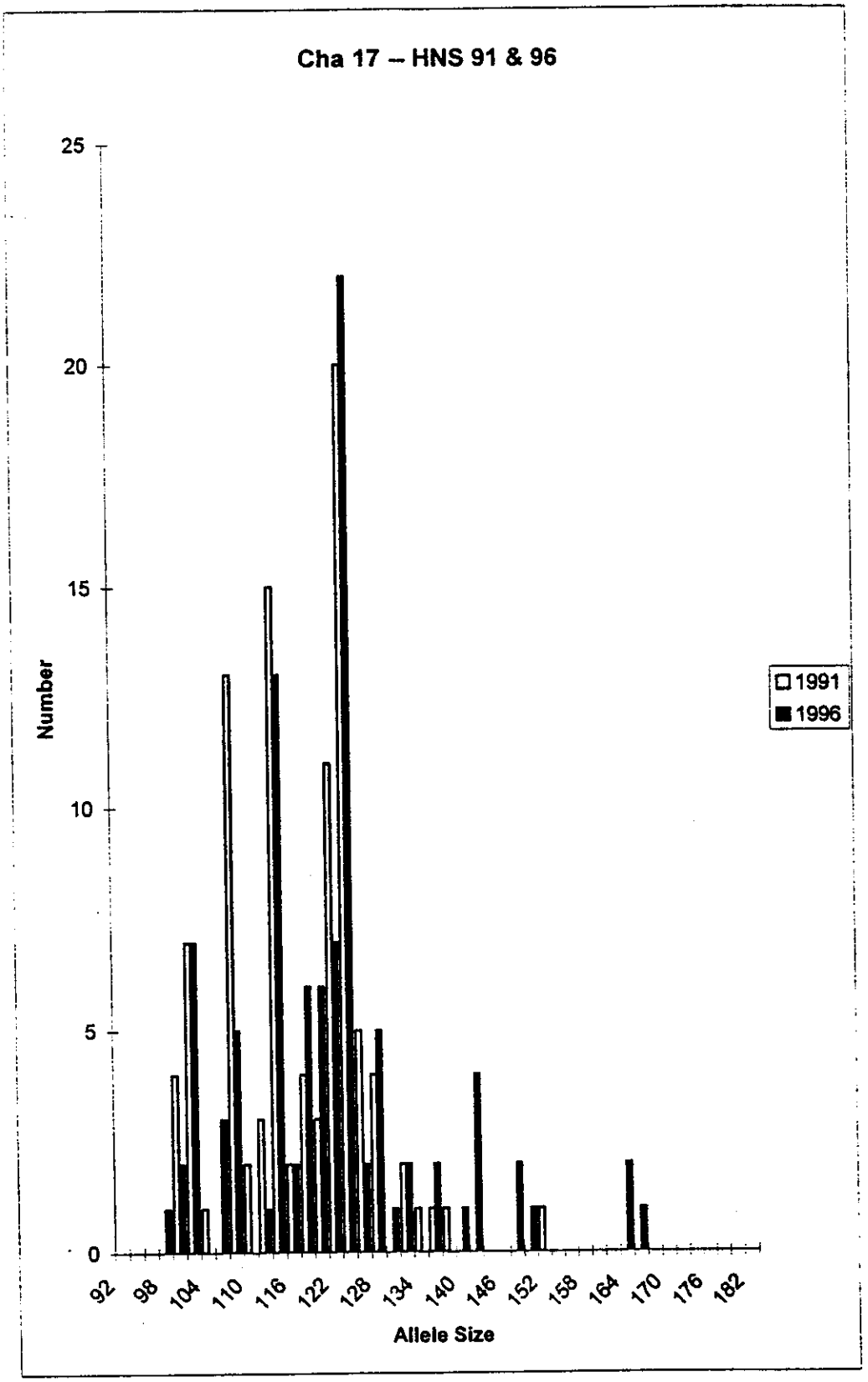


Figure 2. Allele frequencies of Cha 17 for each population sampled over two years.



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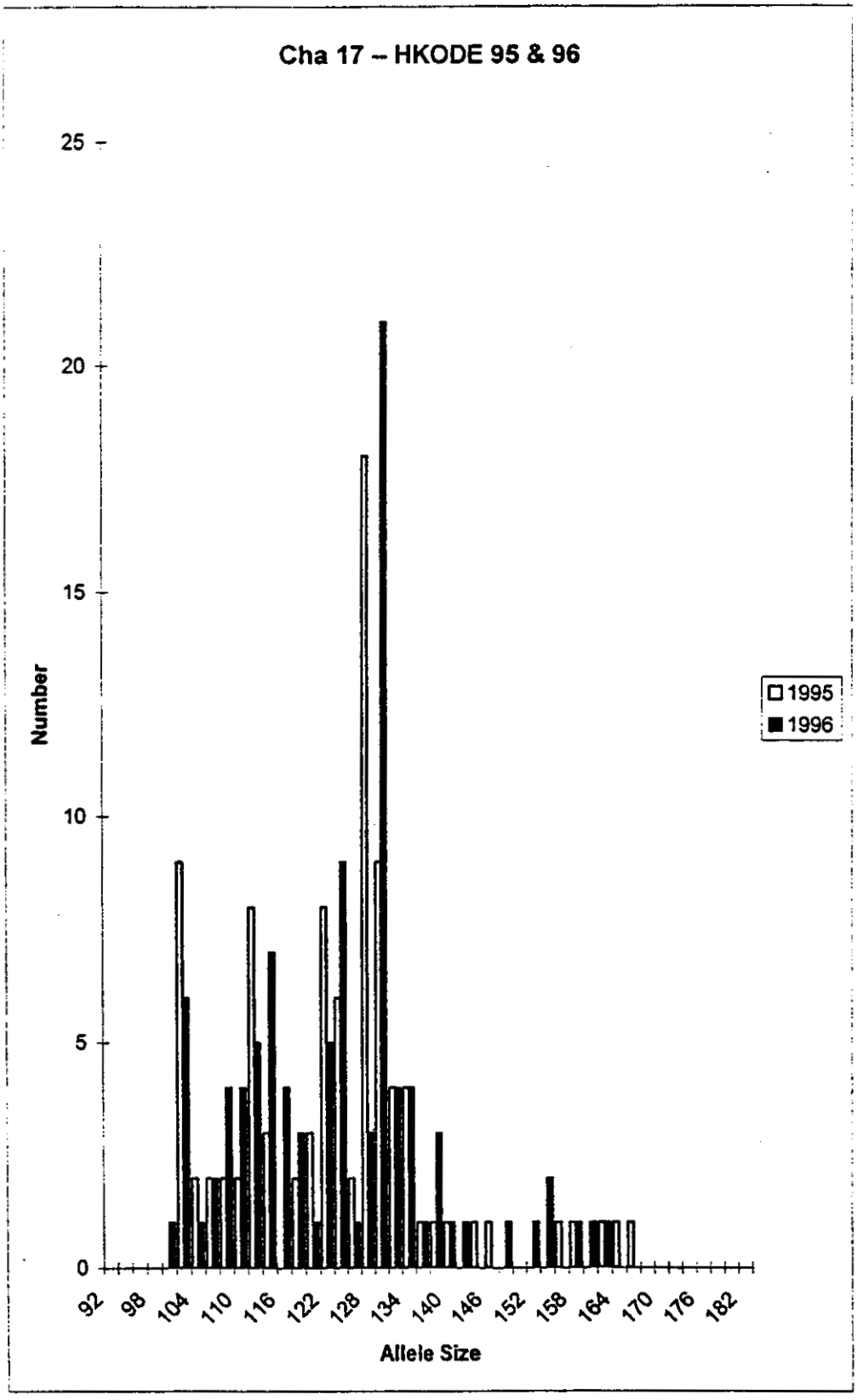

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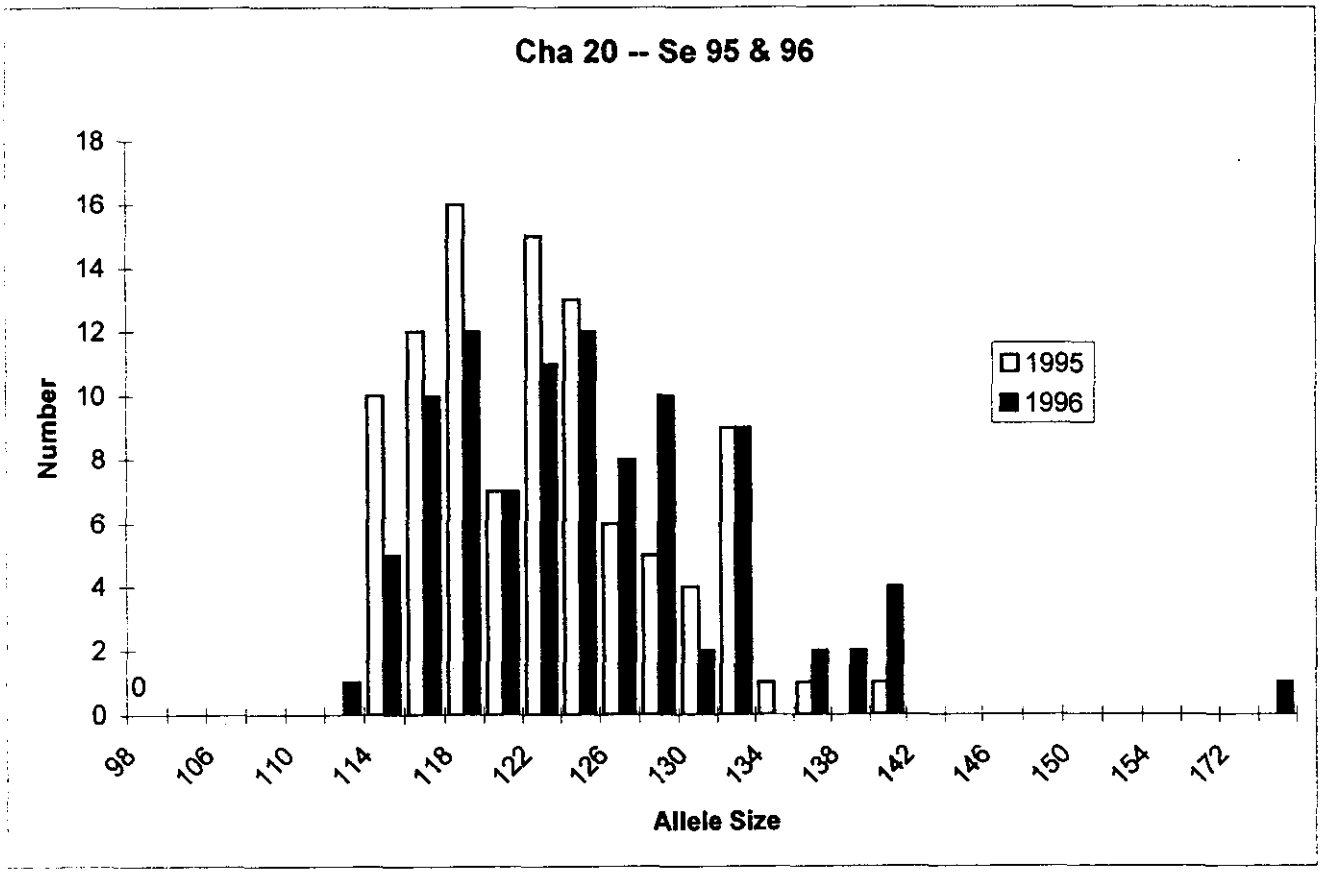


Figure 3. Allele frequencies of Cha 20 for each population sampled over two years.

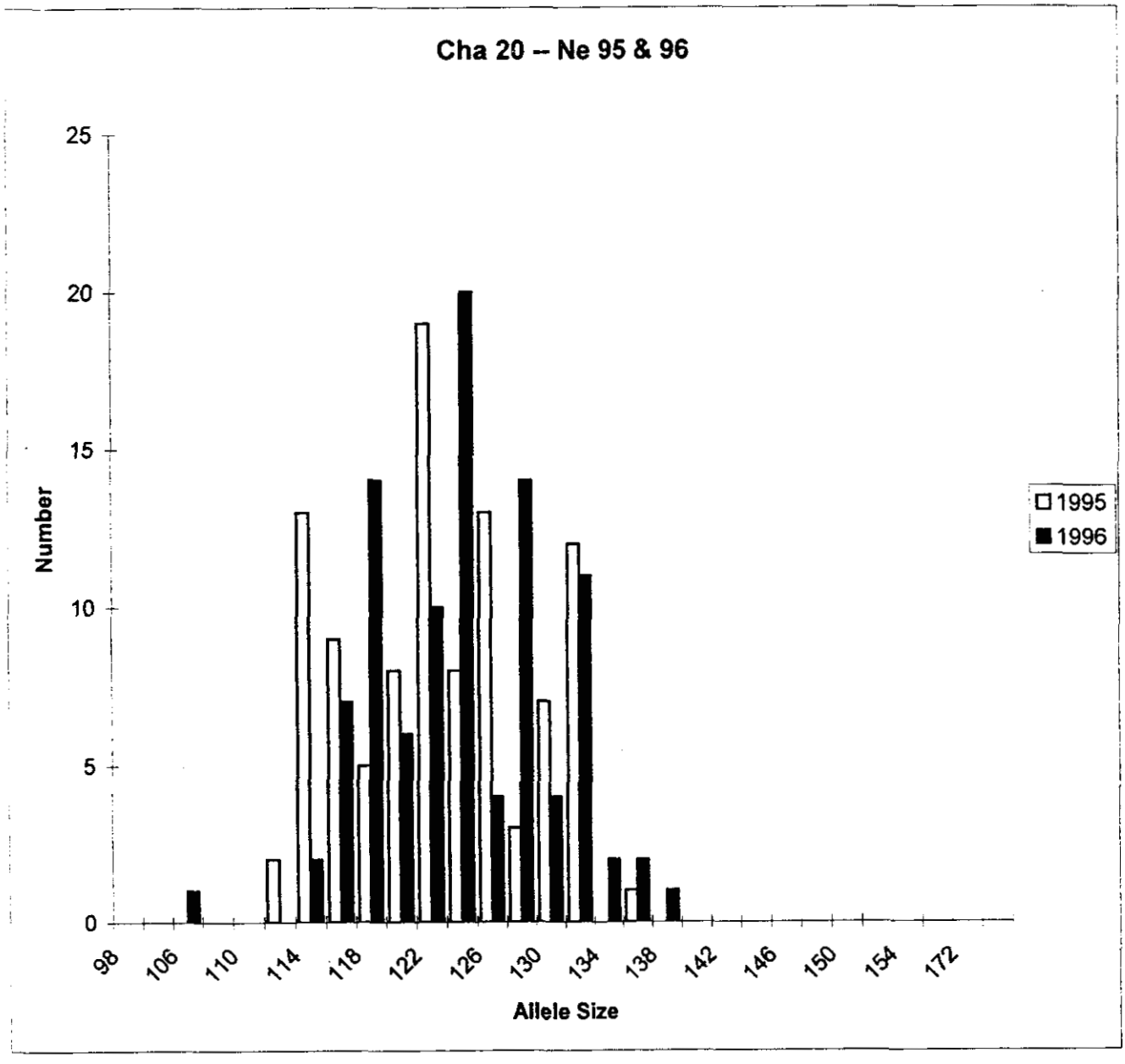
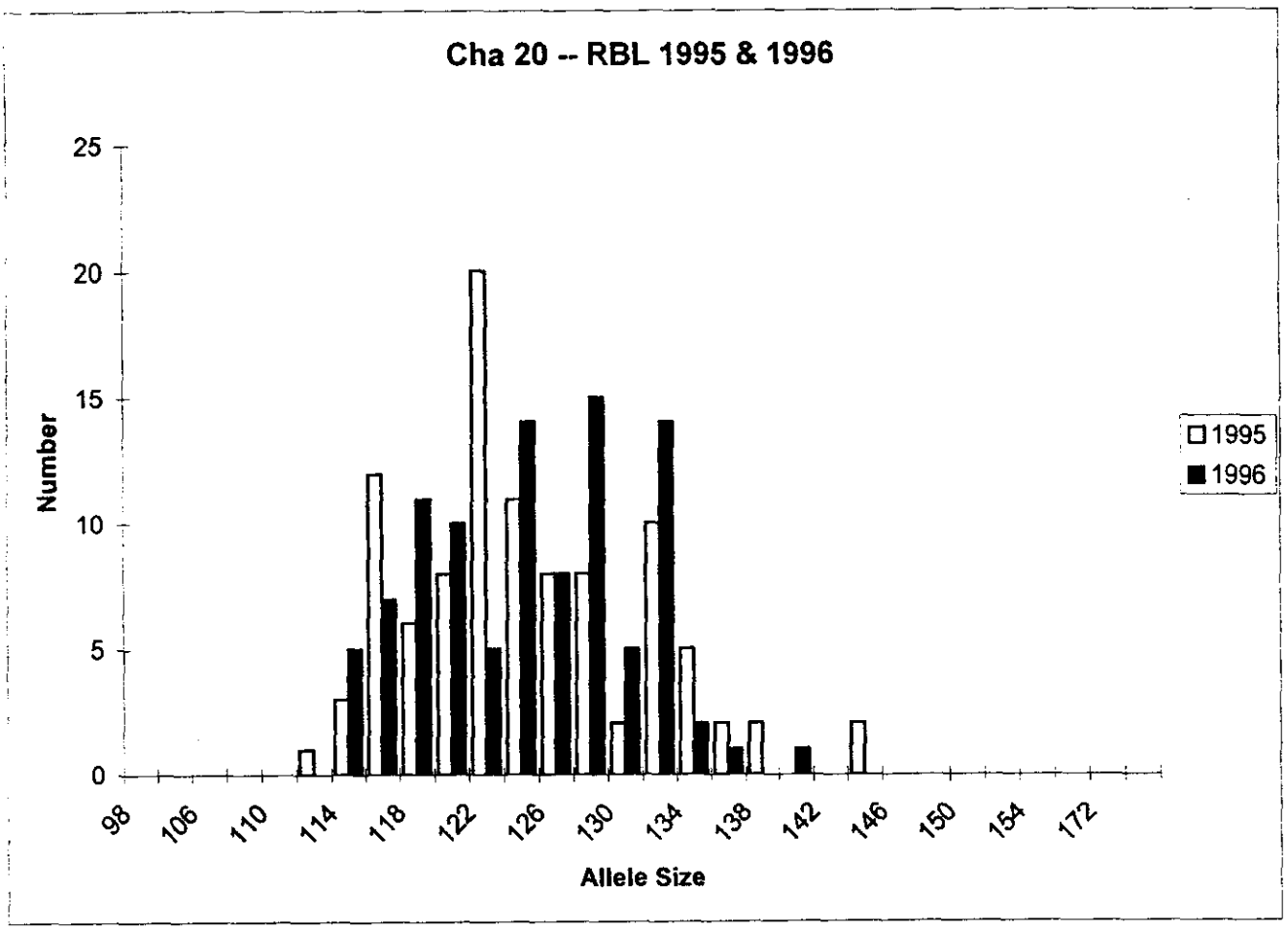


Figure 3. Allele frequencies of Cha 20 for each population sampled over two years.



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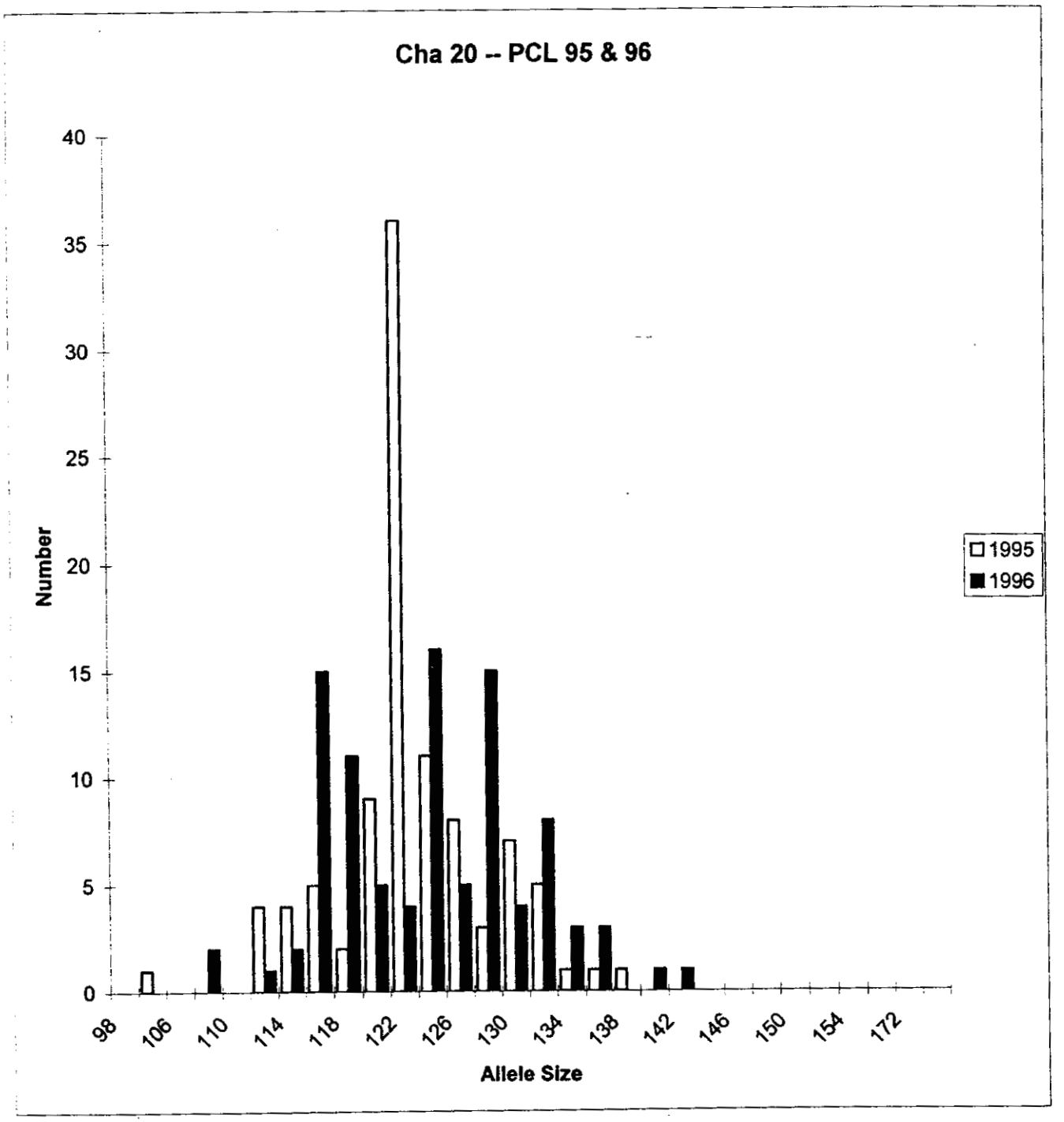

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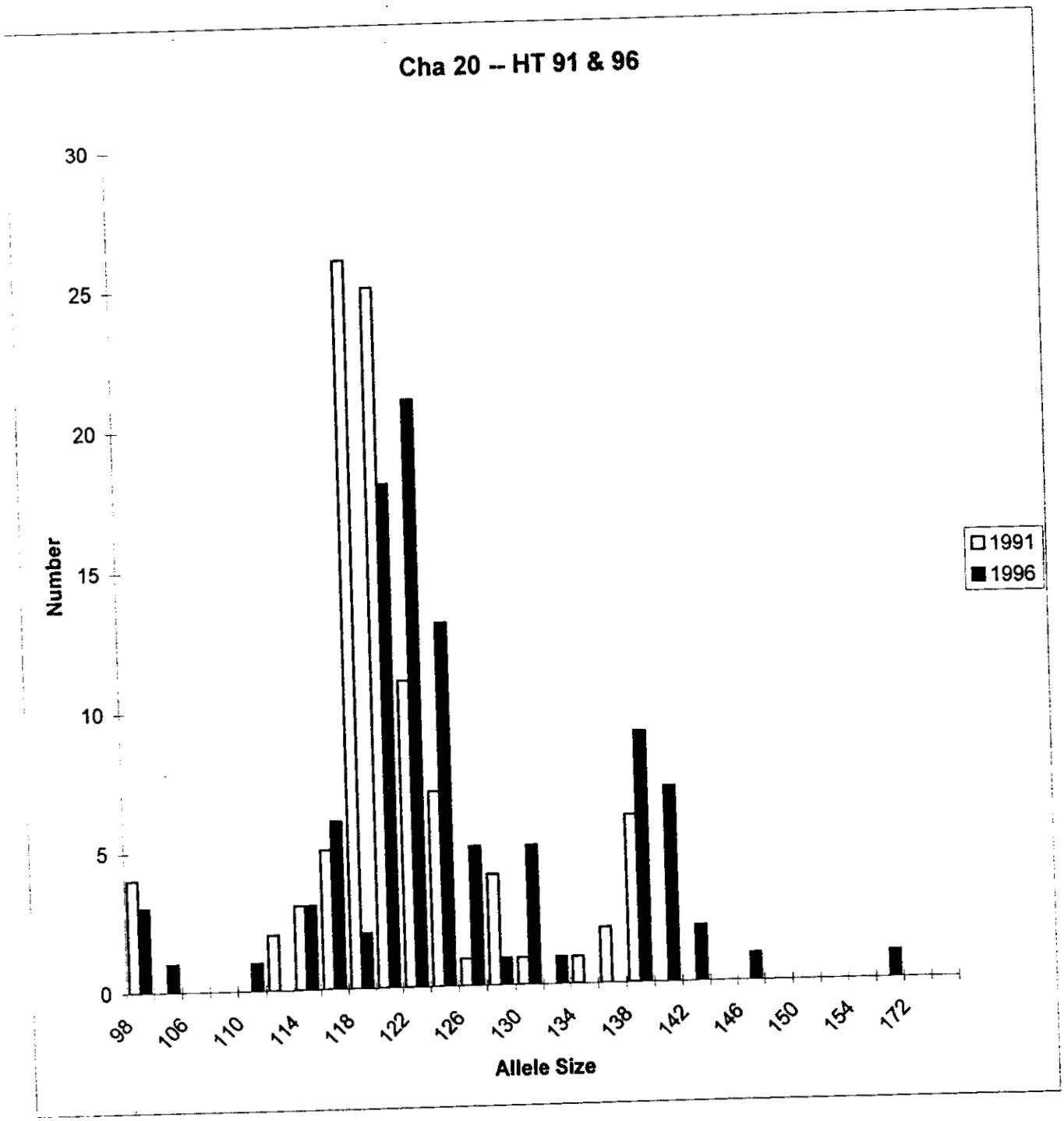

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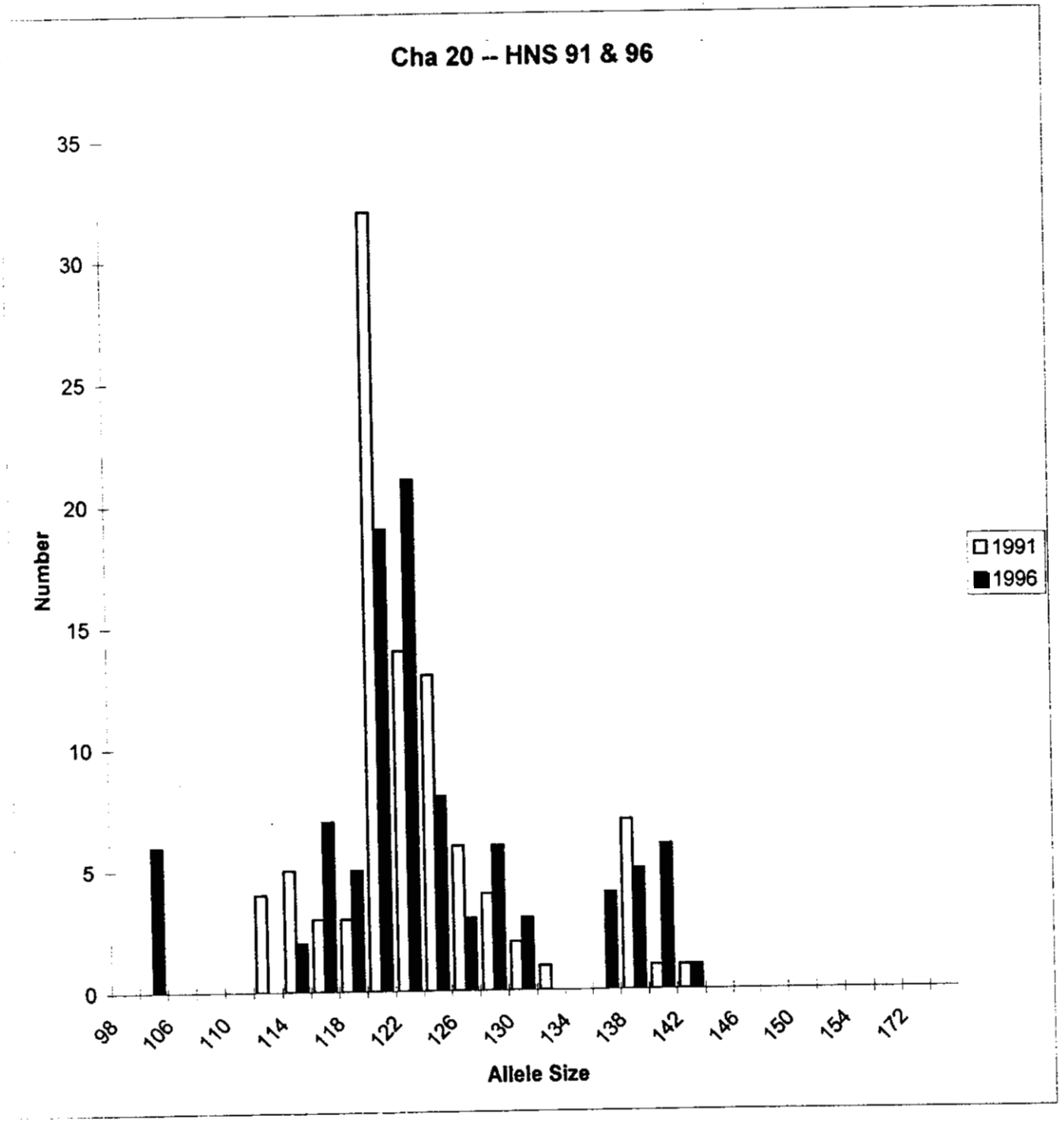

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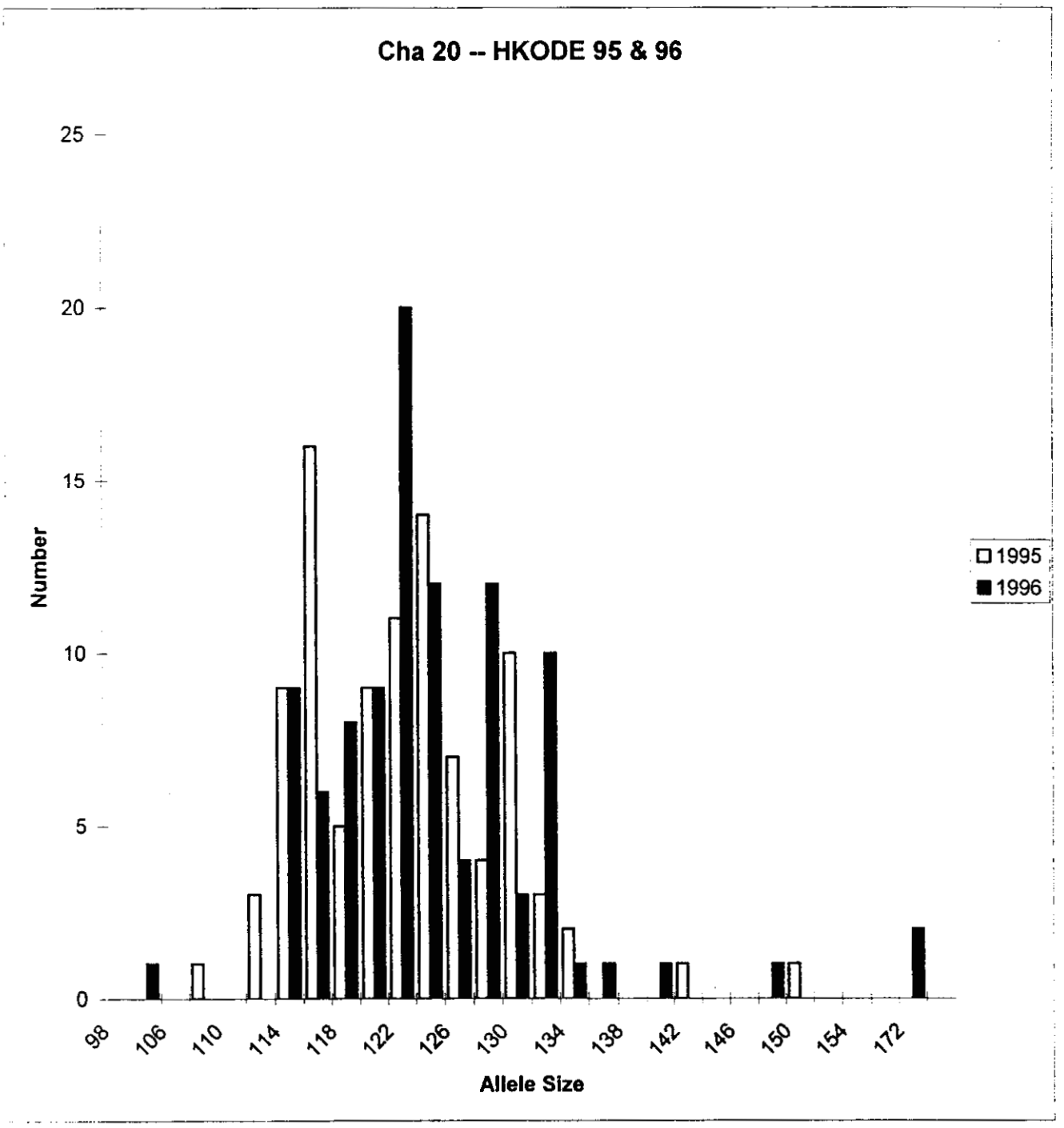


Figure 3. Allele frequencies of Cha 20 for each population sampled over two years.

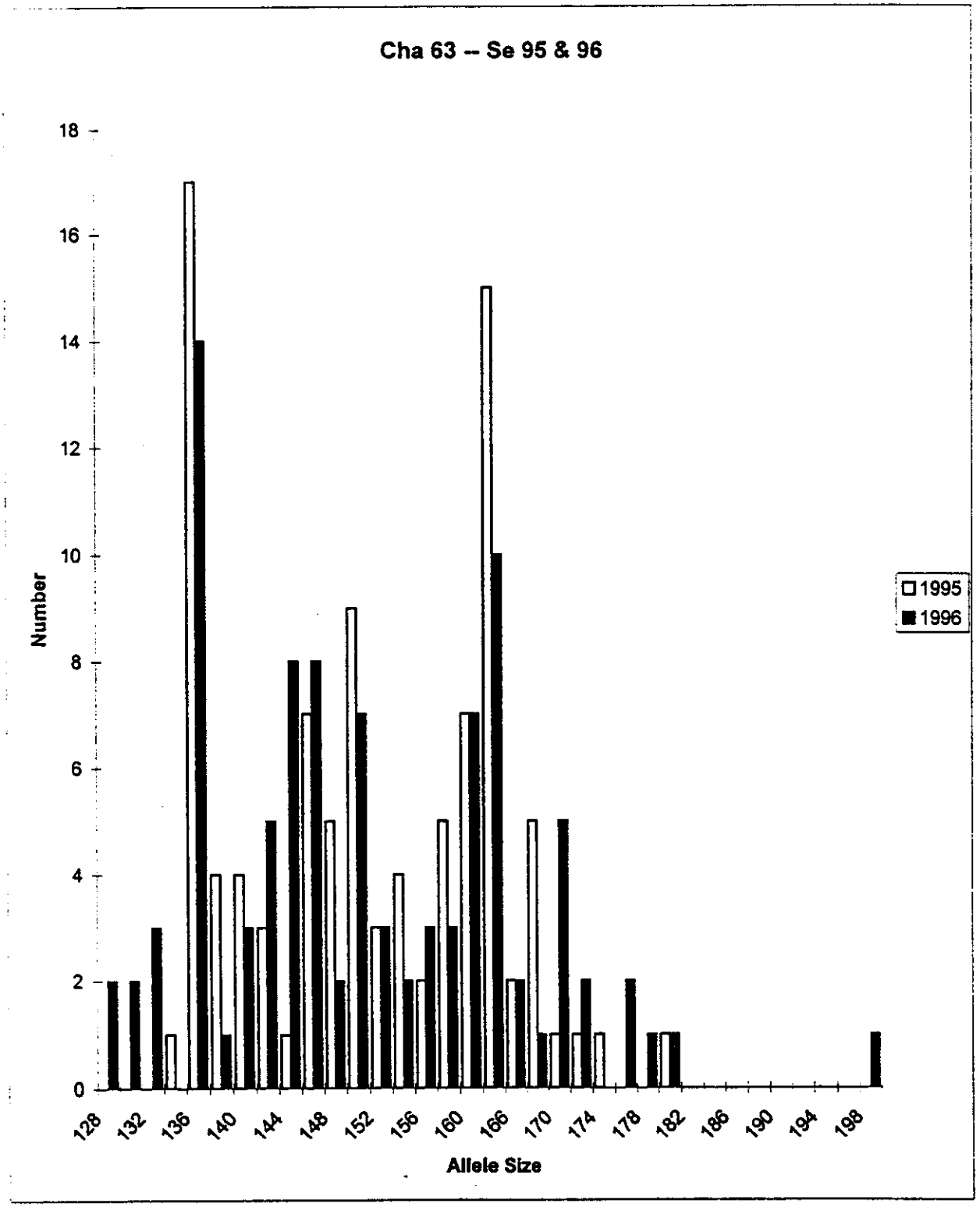
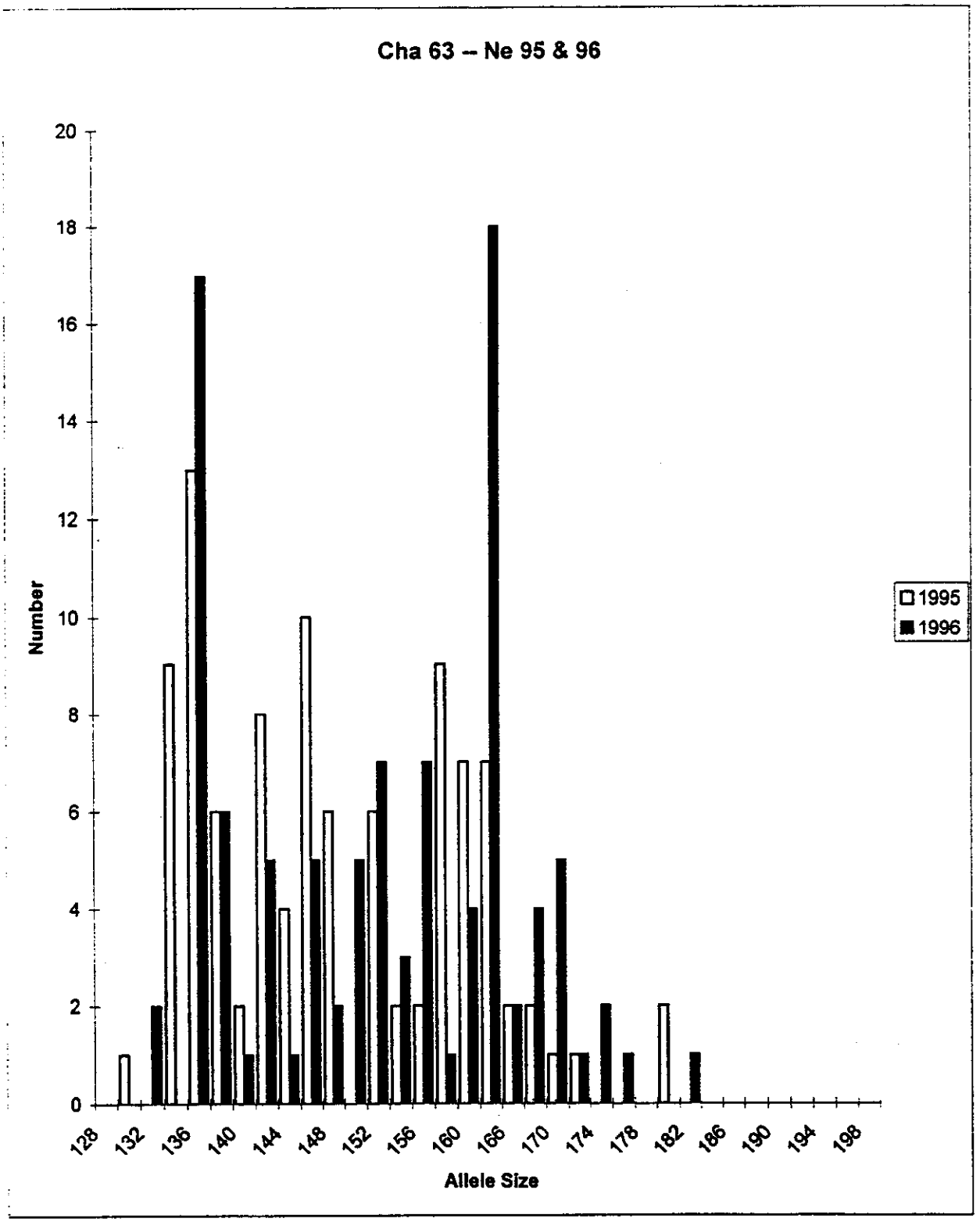


Figure 4. Allele frequencies of Cha 63 for each population sampled over two years.



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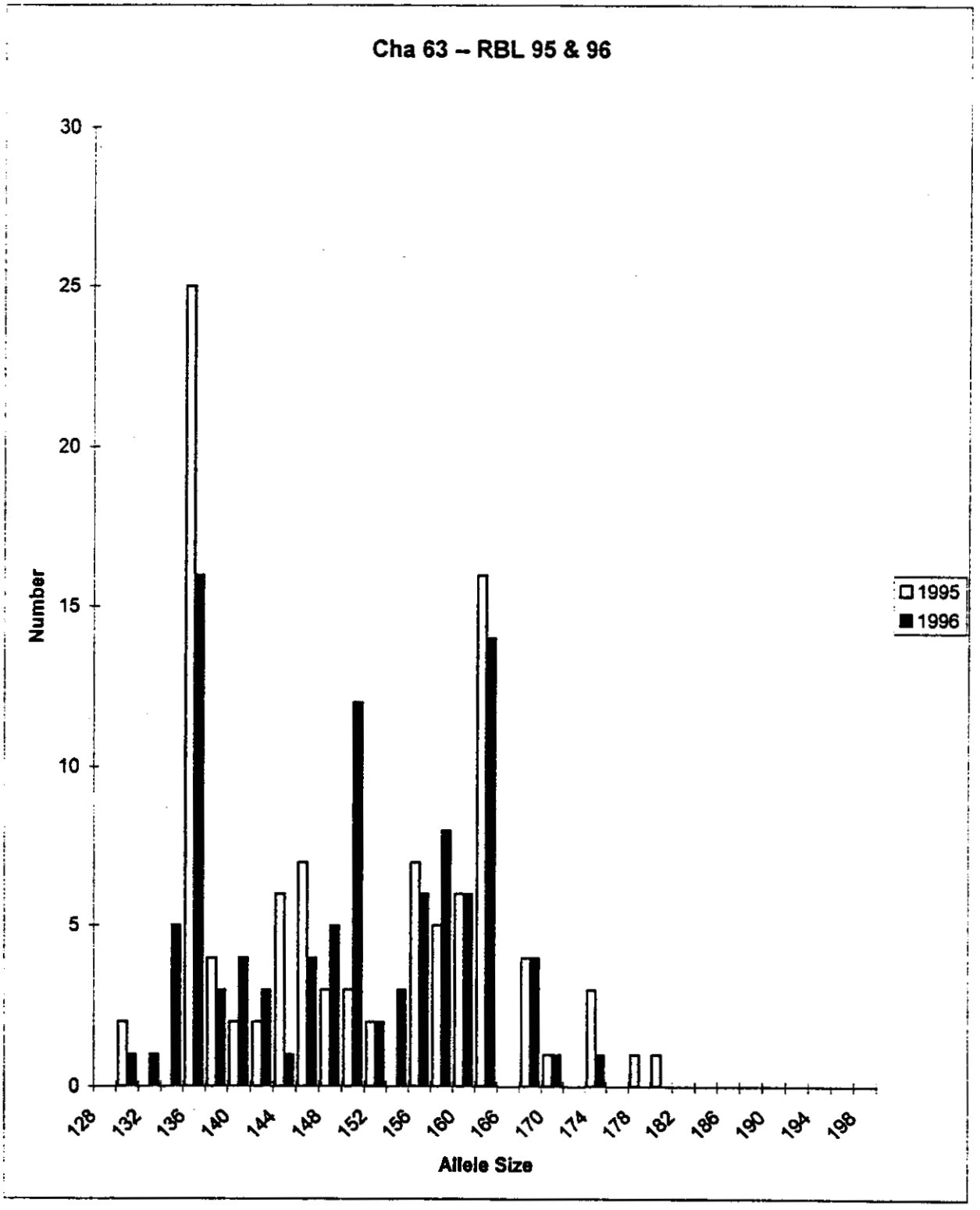

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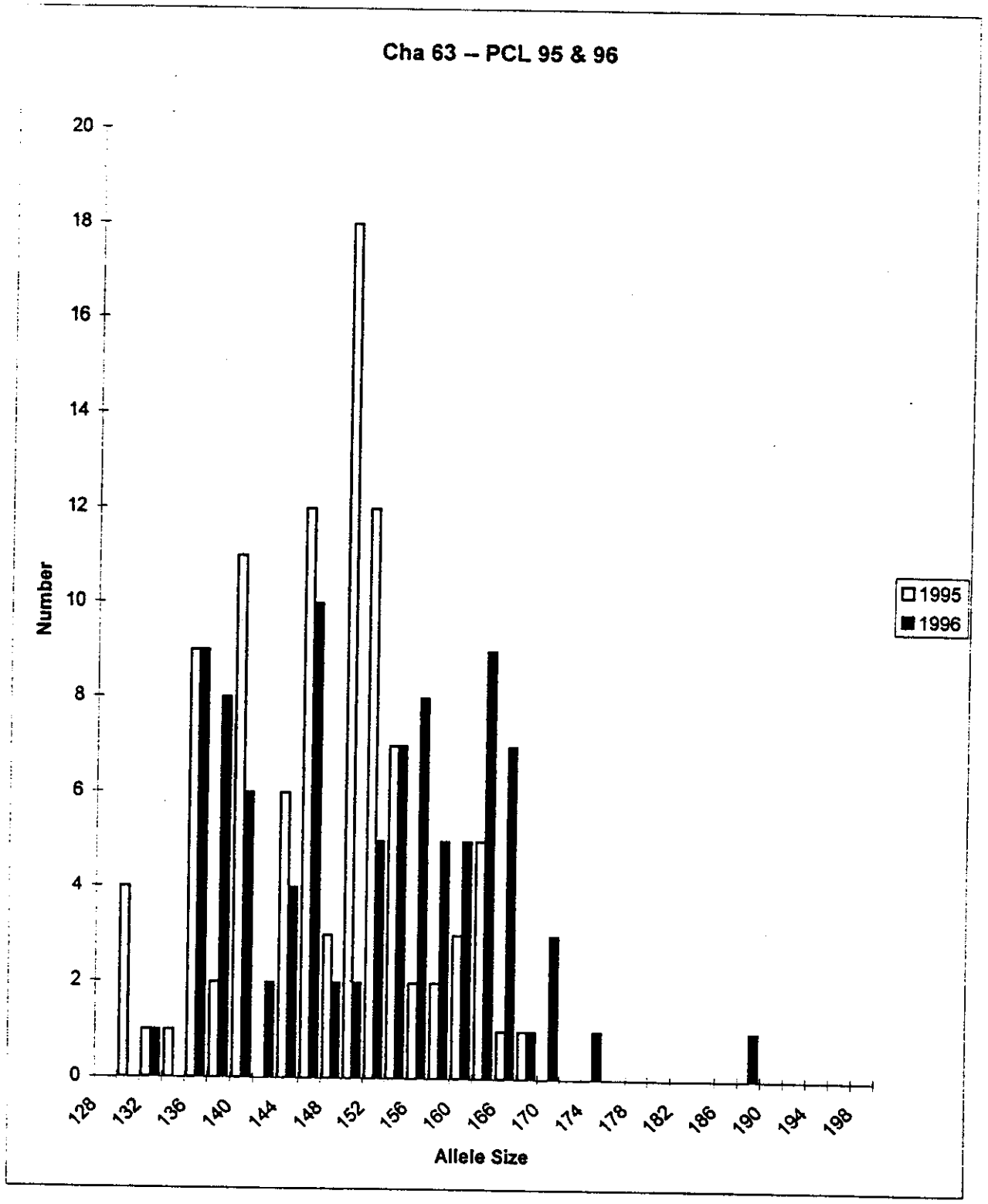

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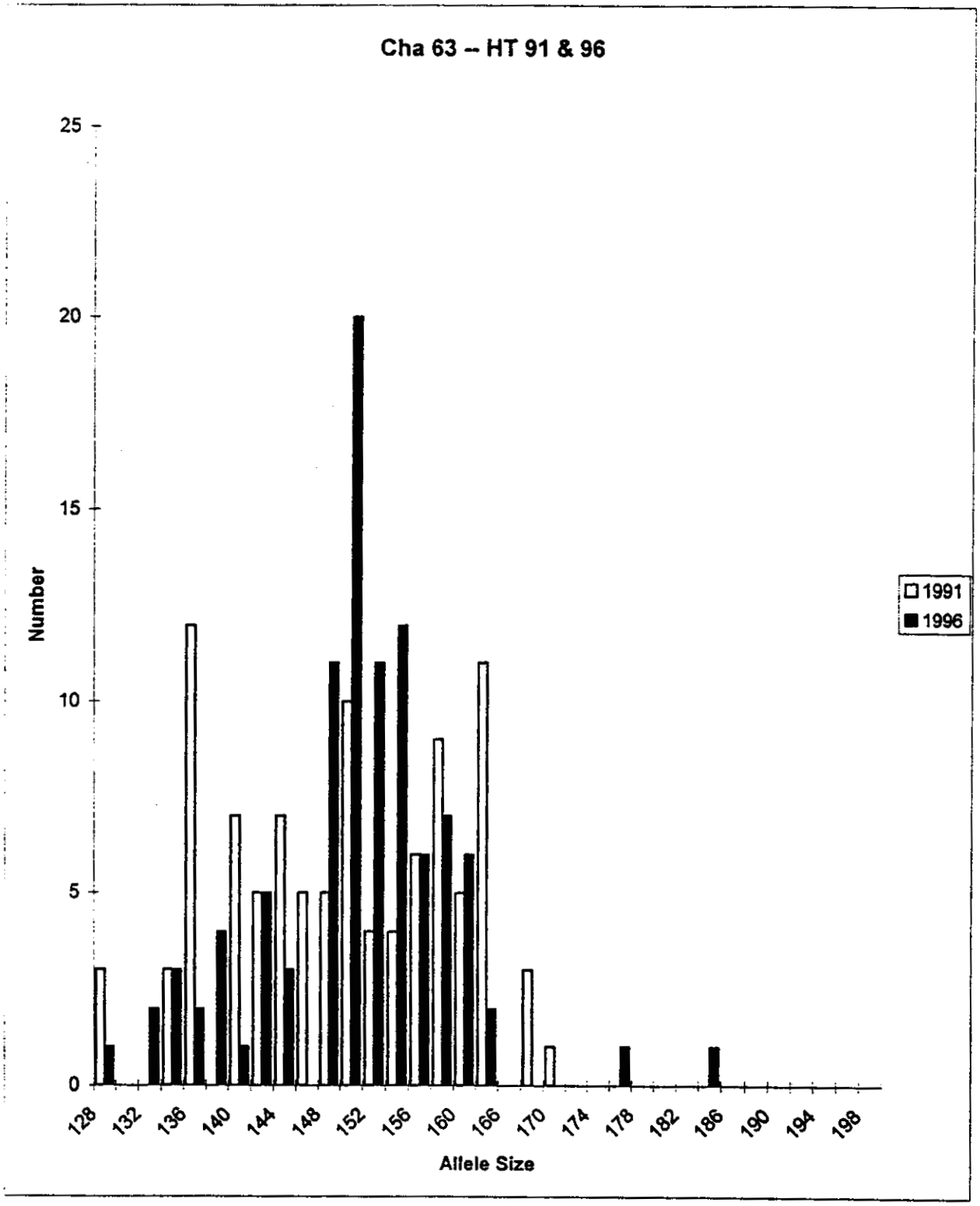

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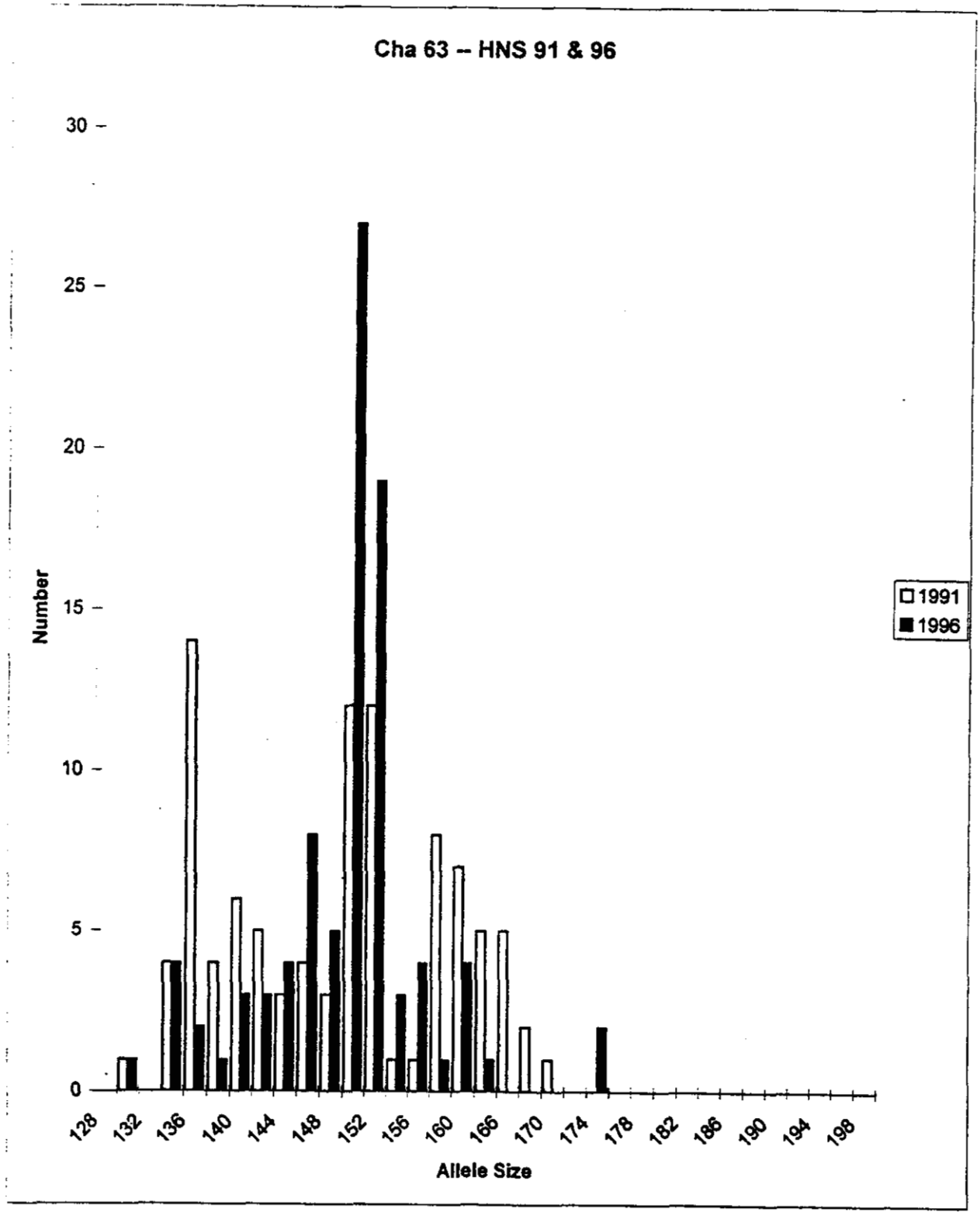

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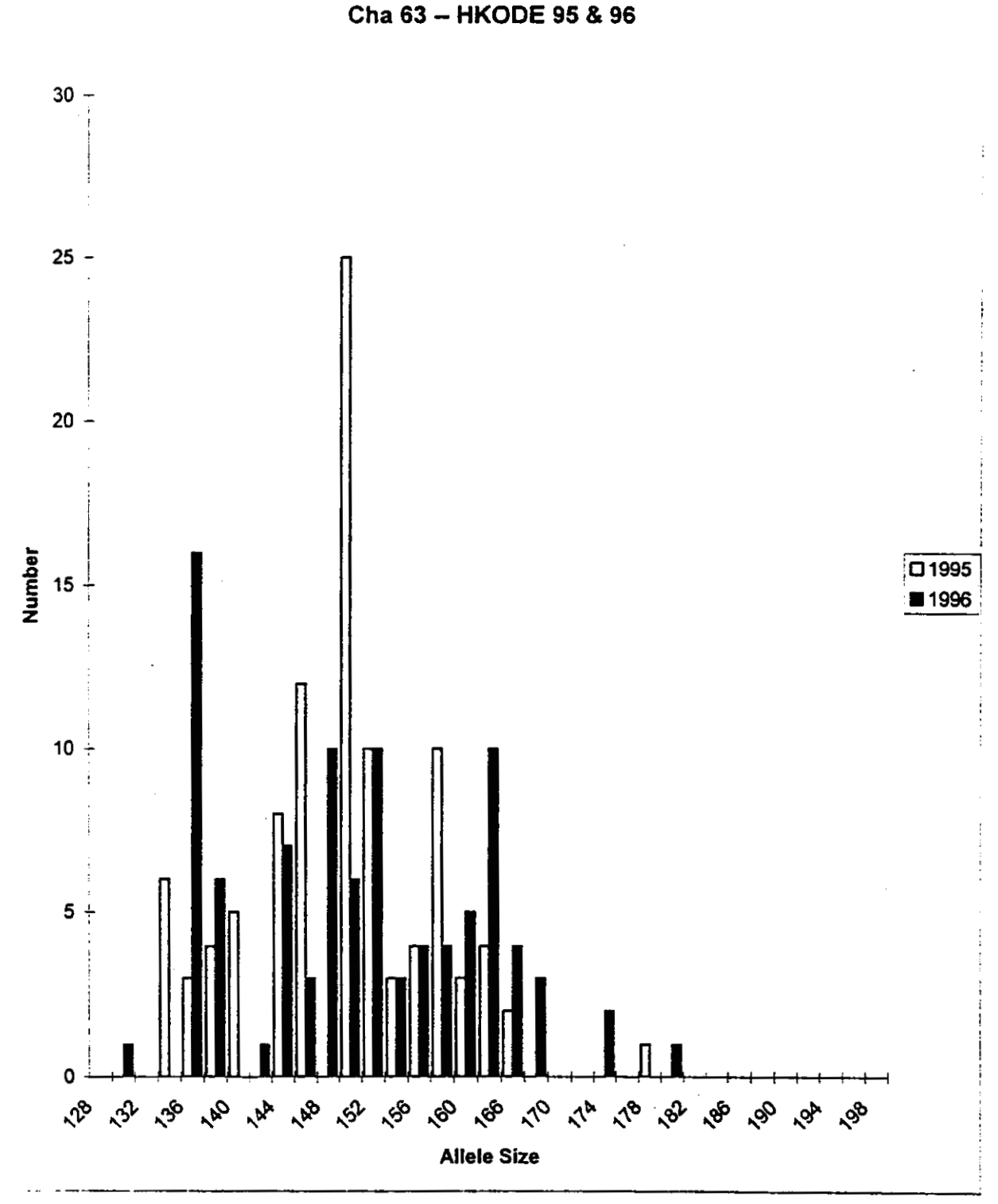

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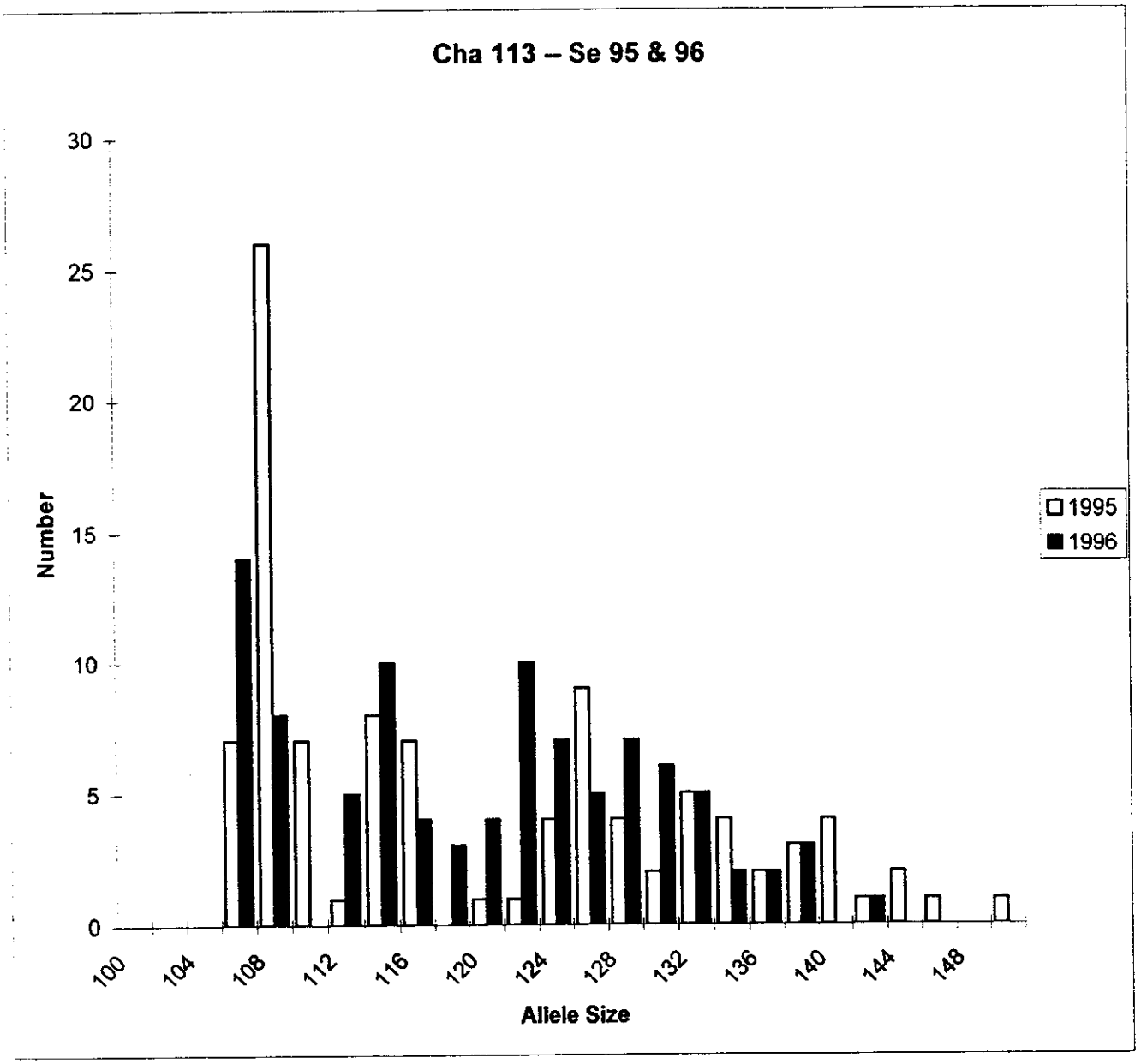
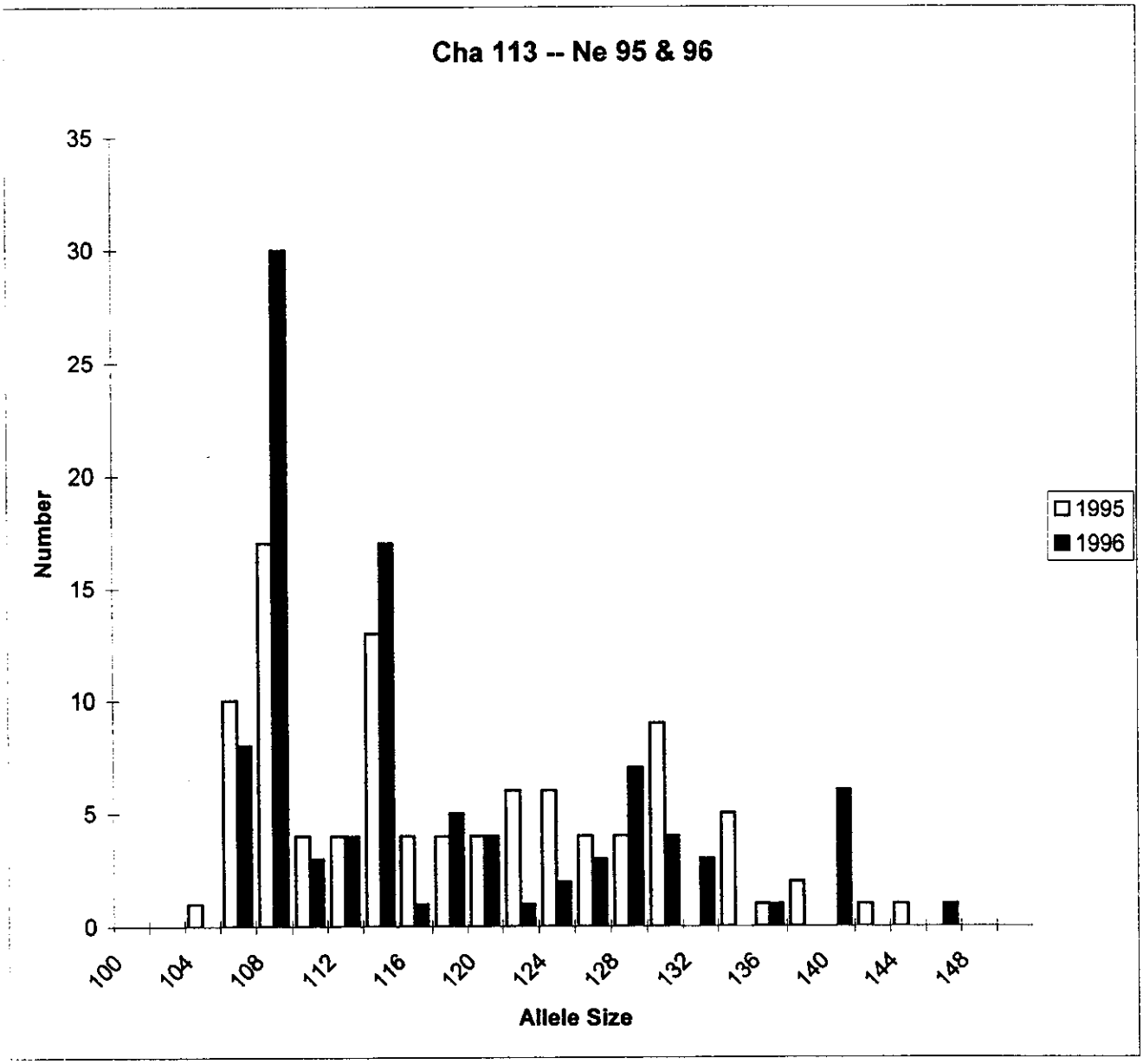


Figure 5. Allele frequencies of Cha 113 for each population sampled over two years.



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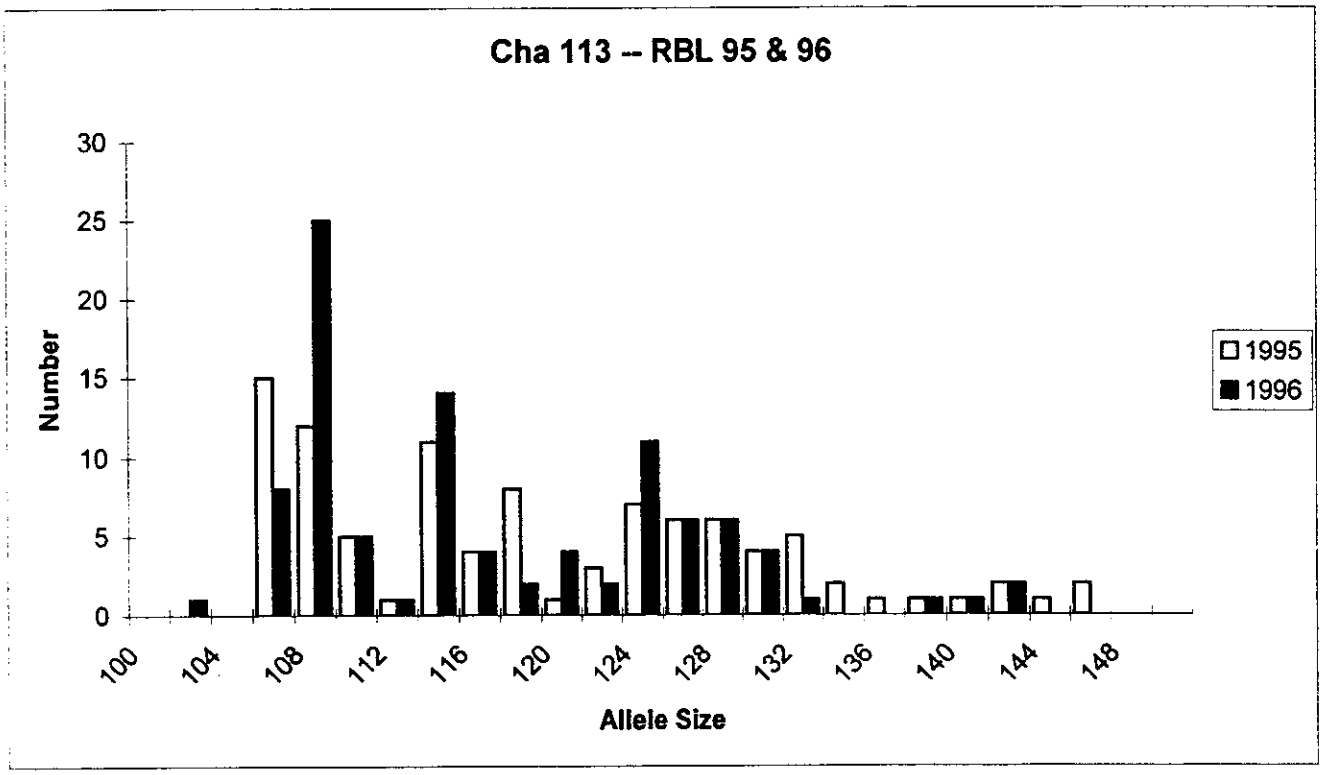

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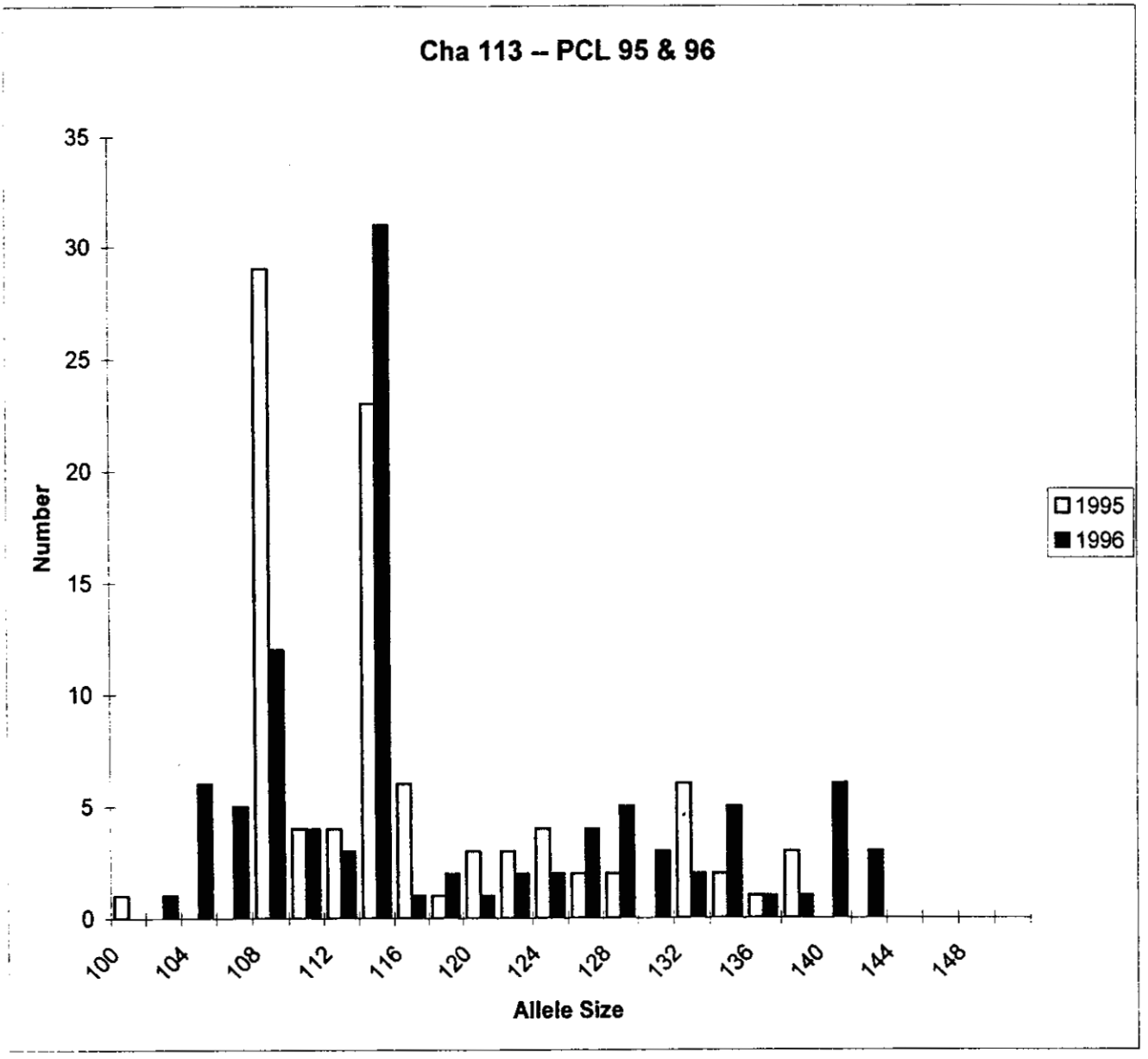
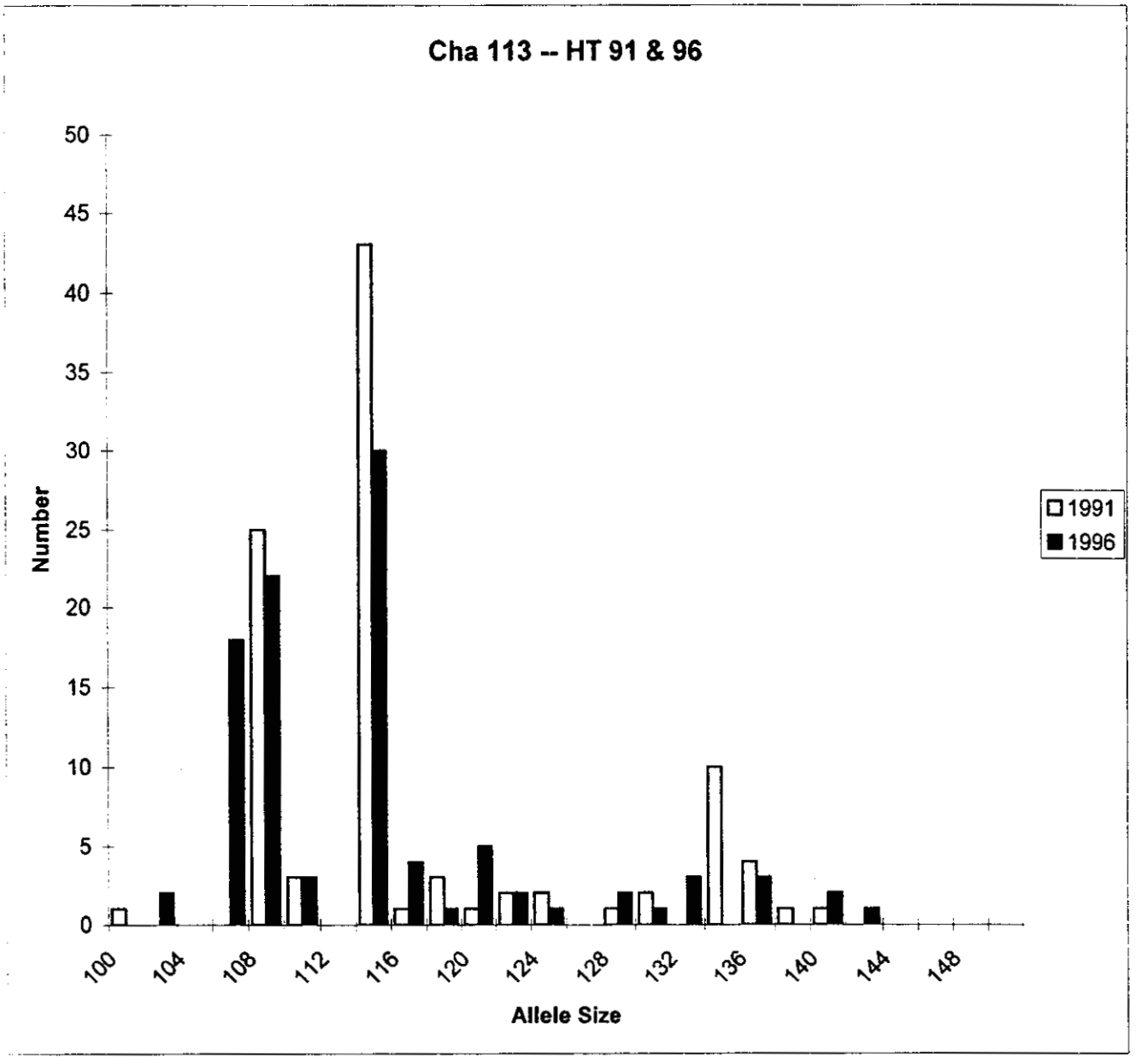


Figure 5. Allele frequencies of Cha 113 for each population sampled over two years.



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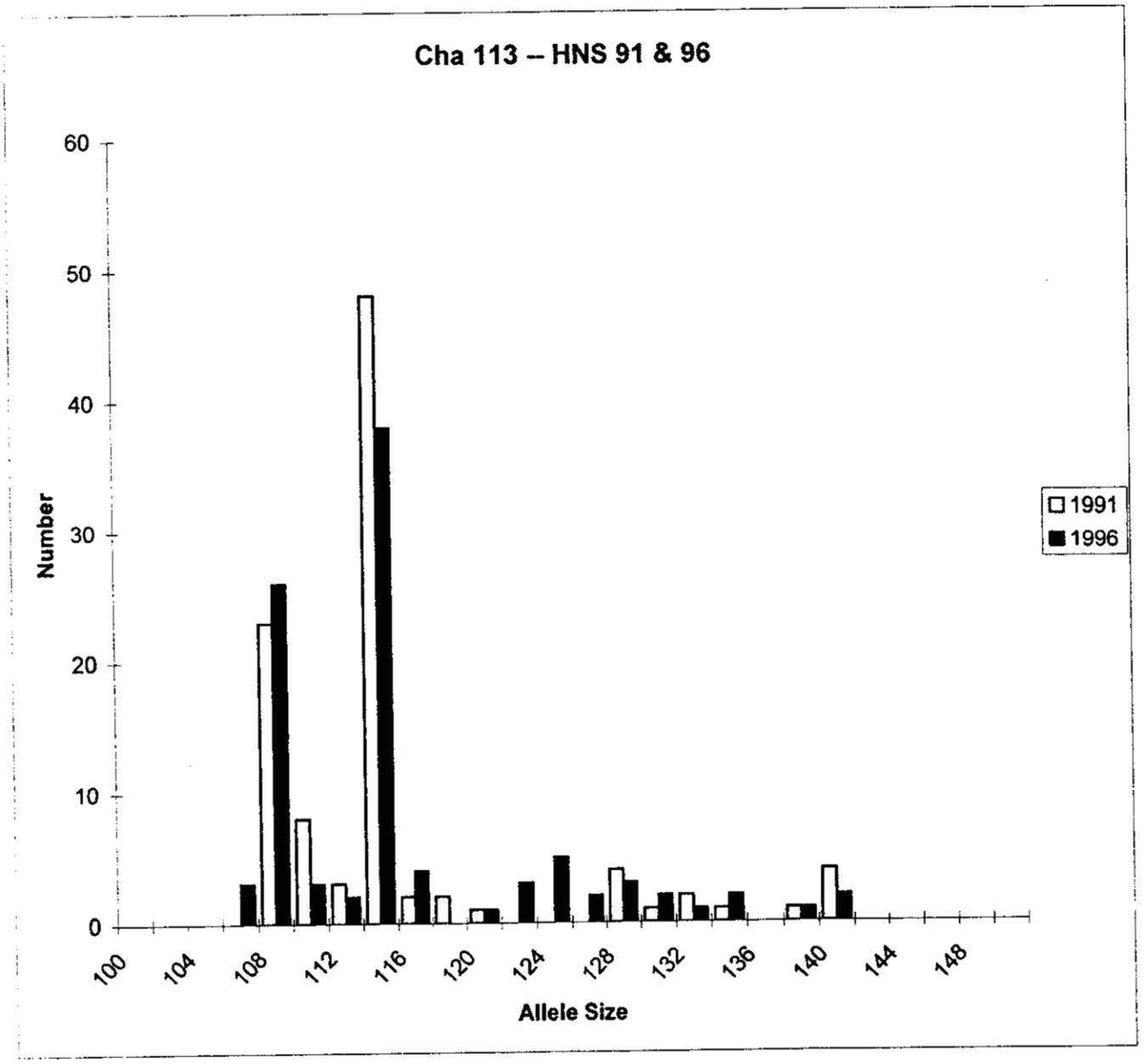

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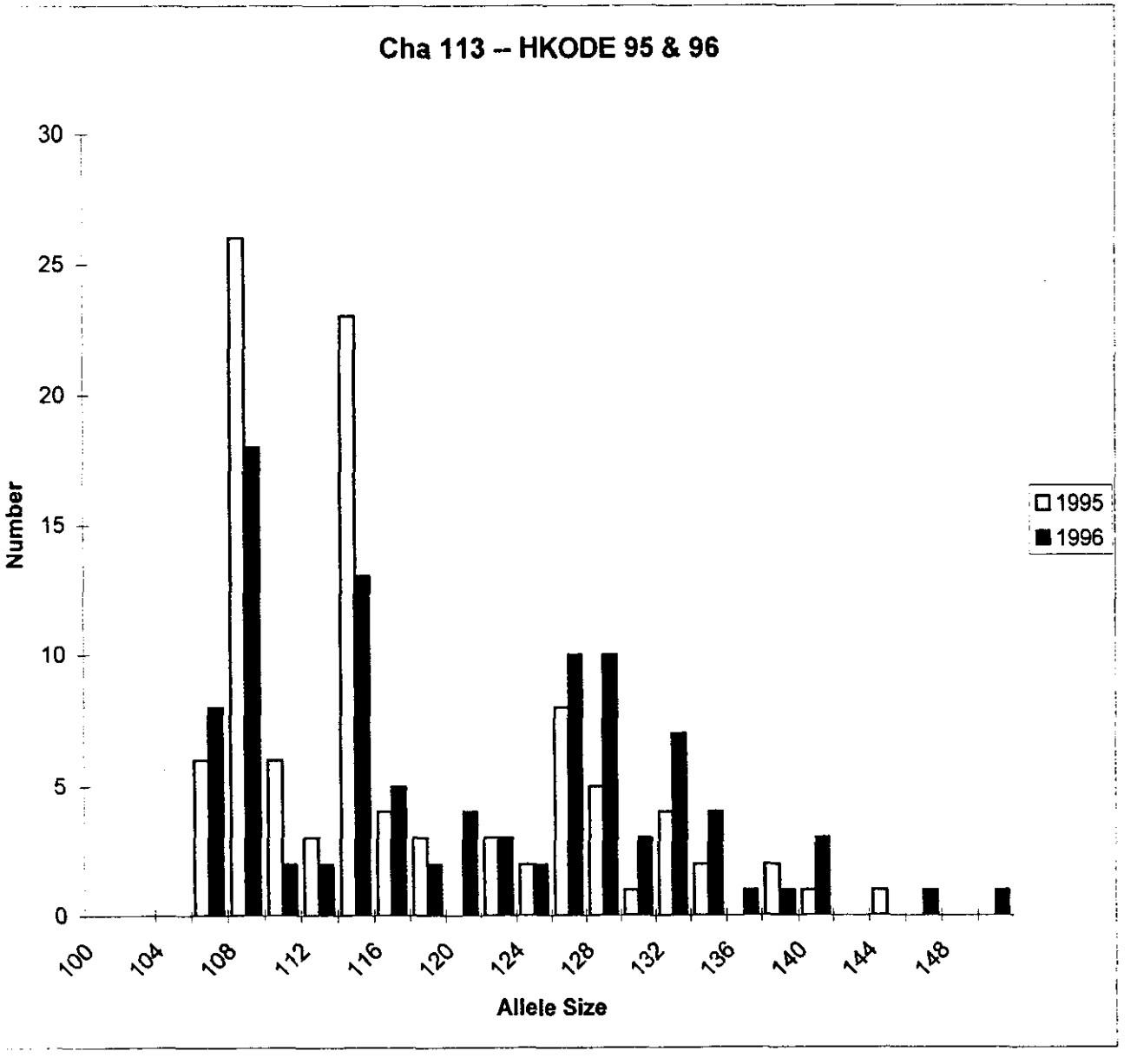


Figure 5. Allele frequencies of Cha 113 for each population sampled over two years.

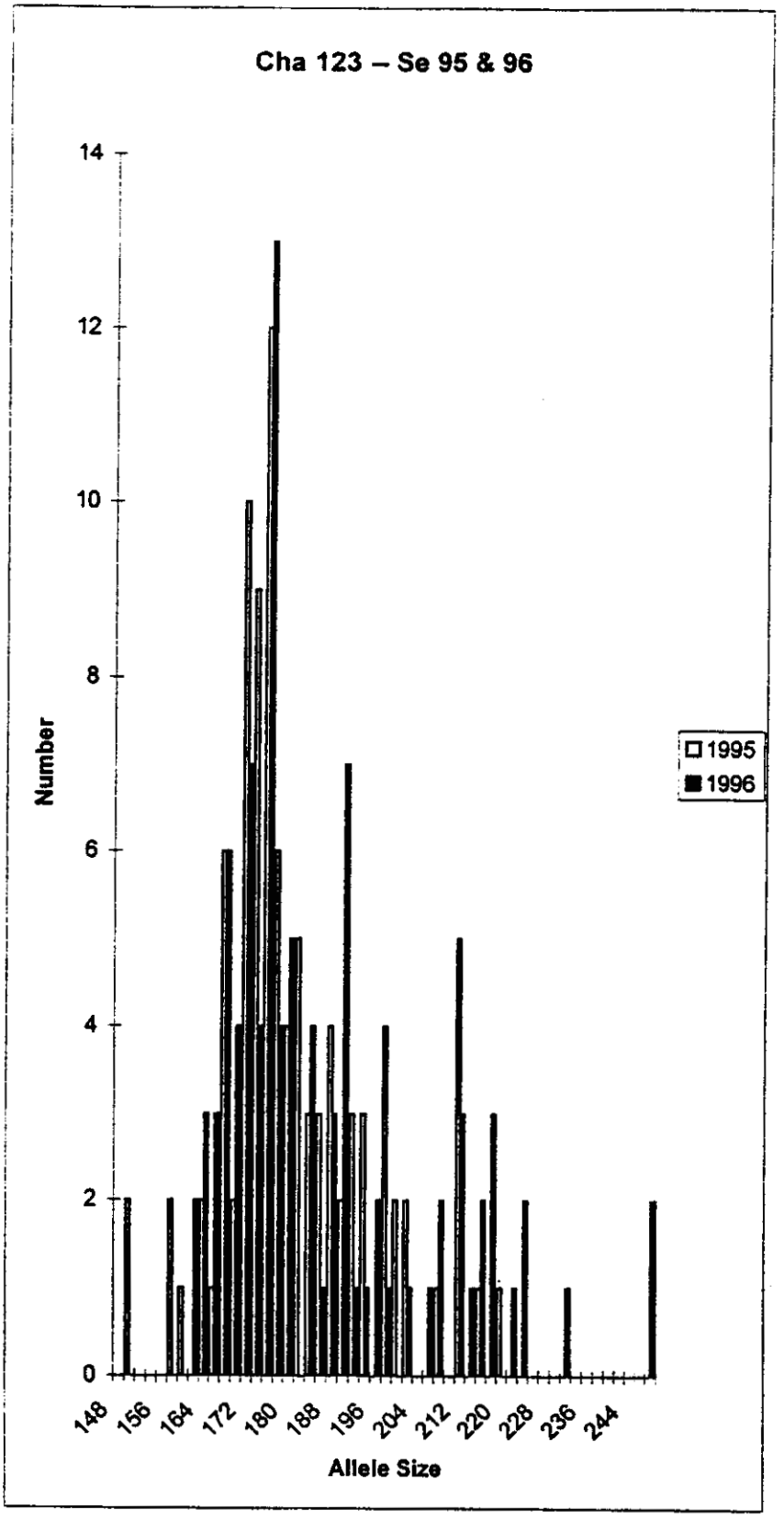
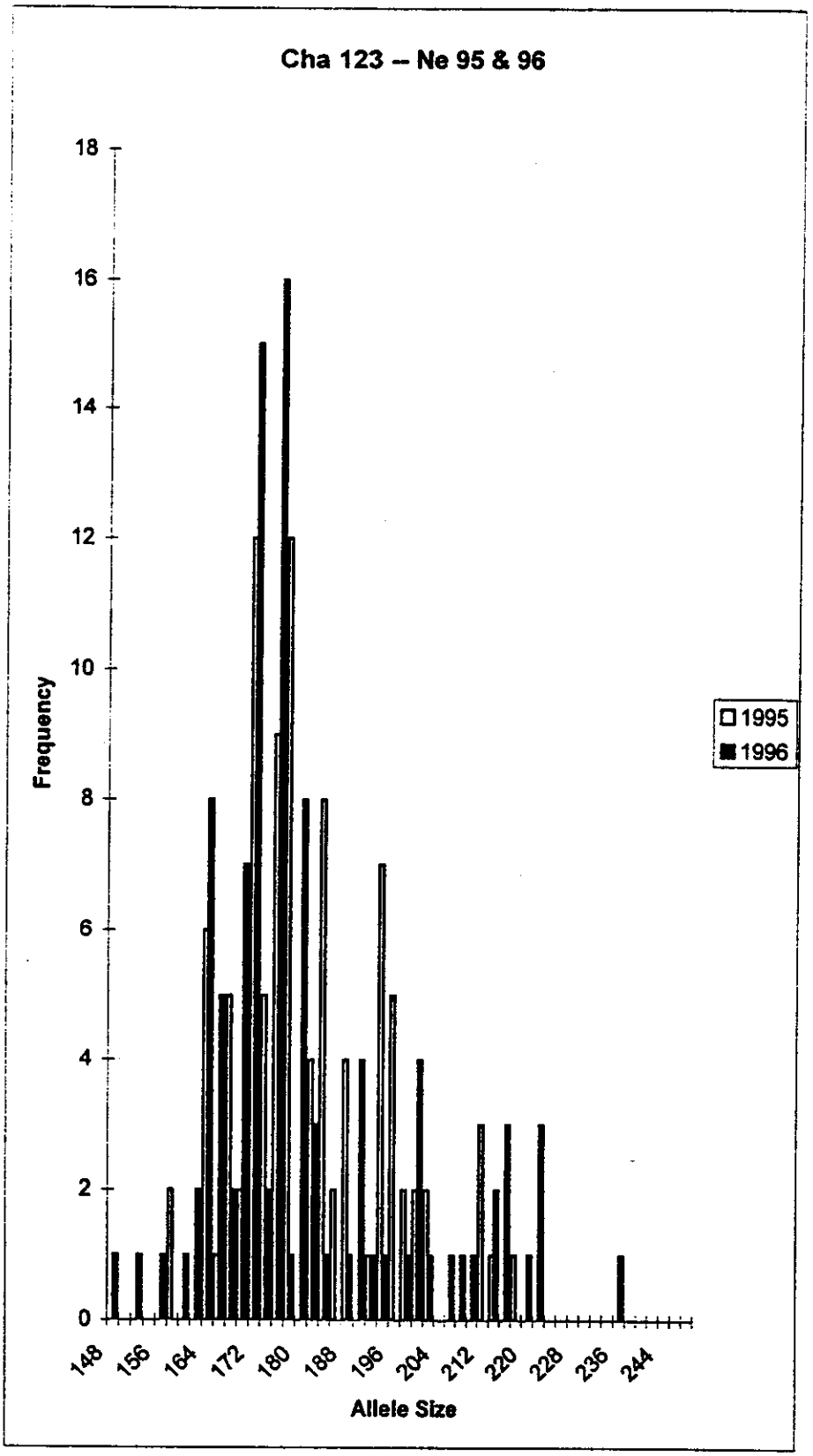


Figure 6. Allele frequencies of Cha 123 for each population sampled over two years.



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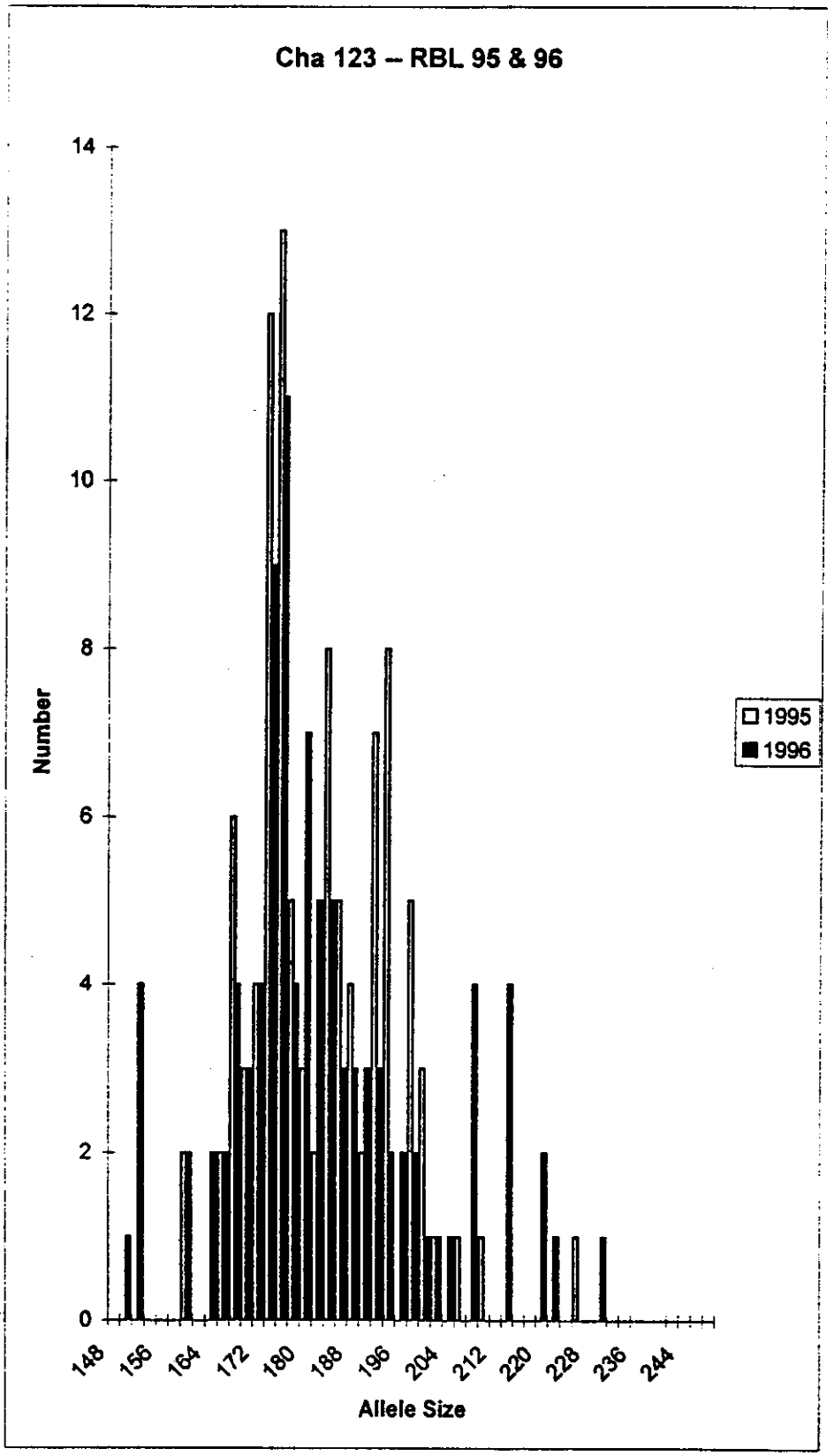

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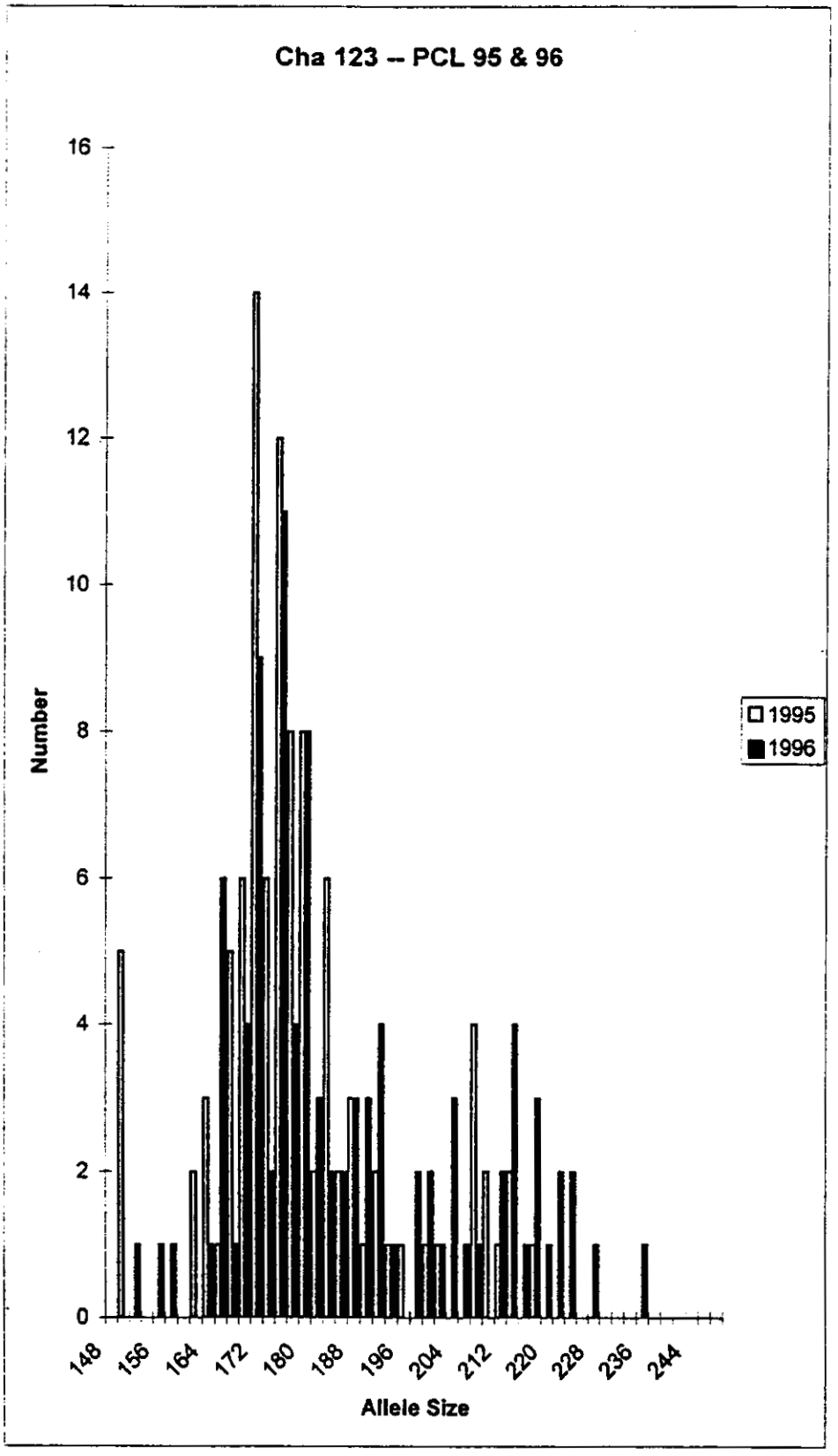

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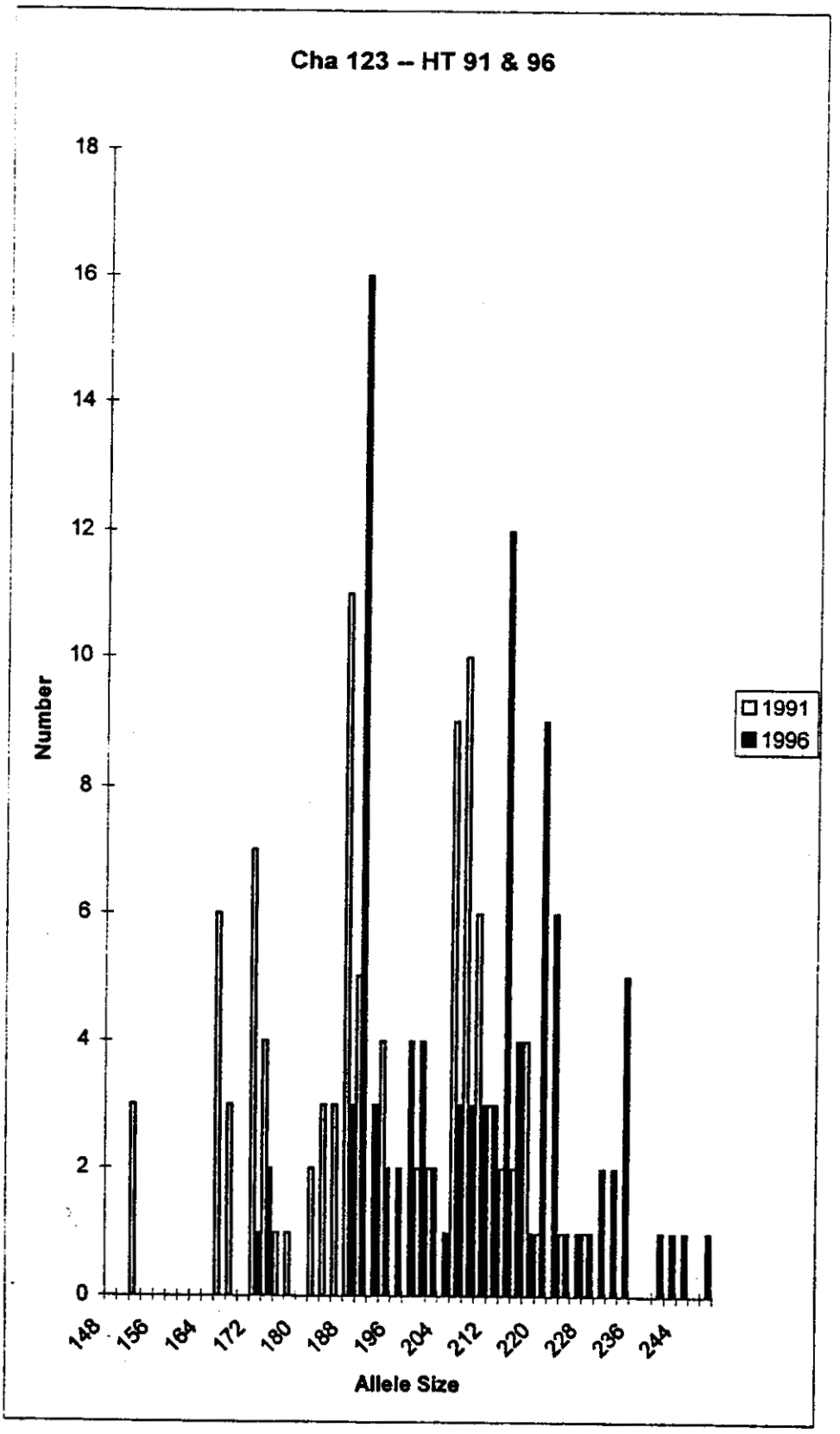

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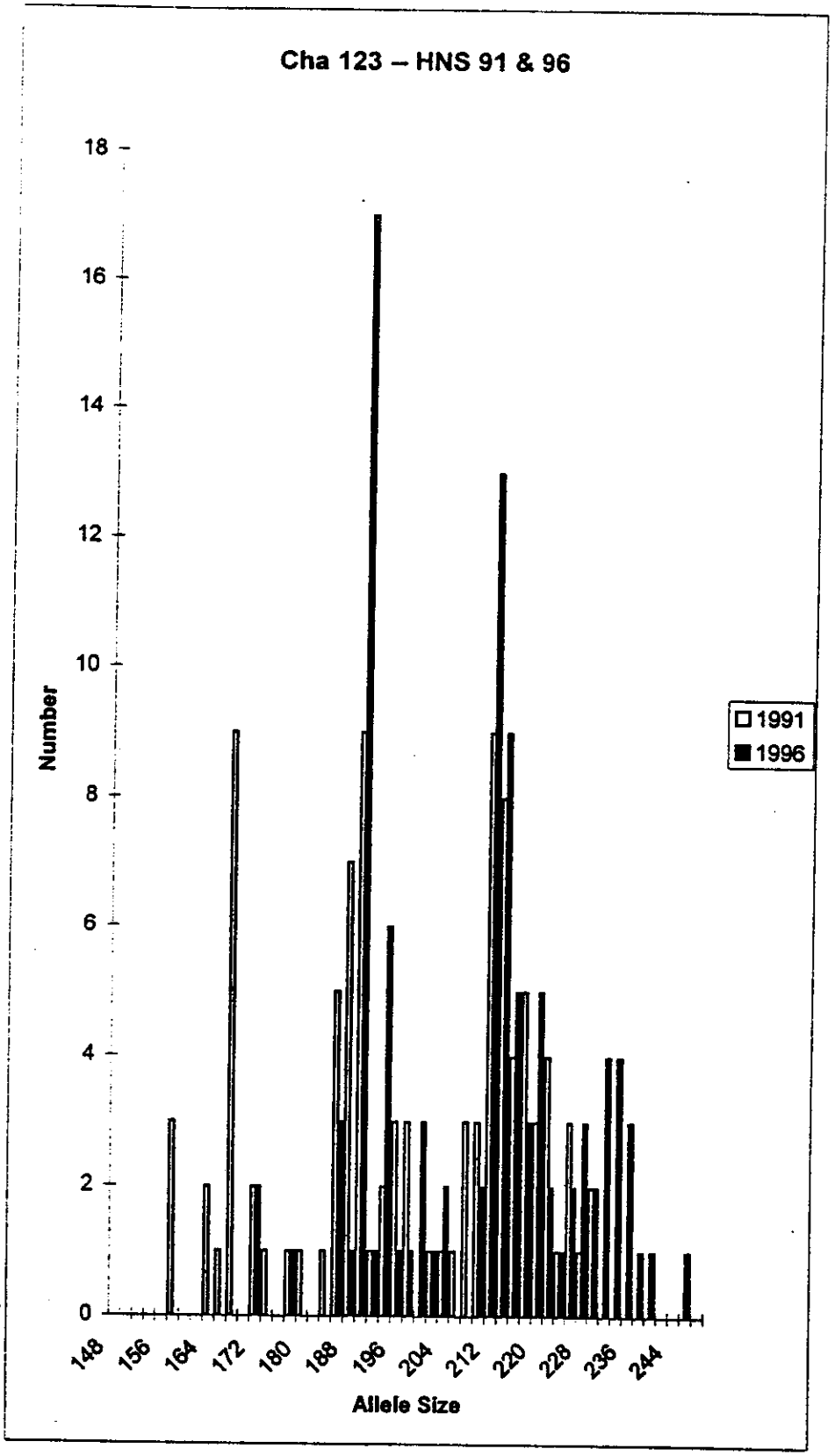

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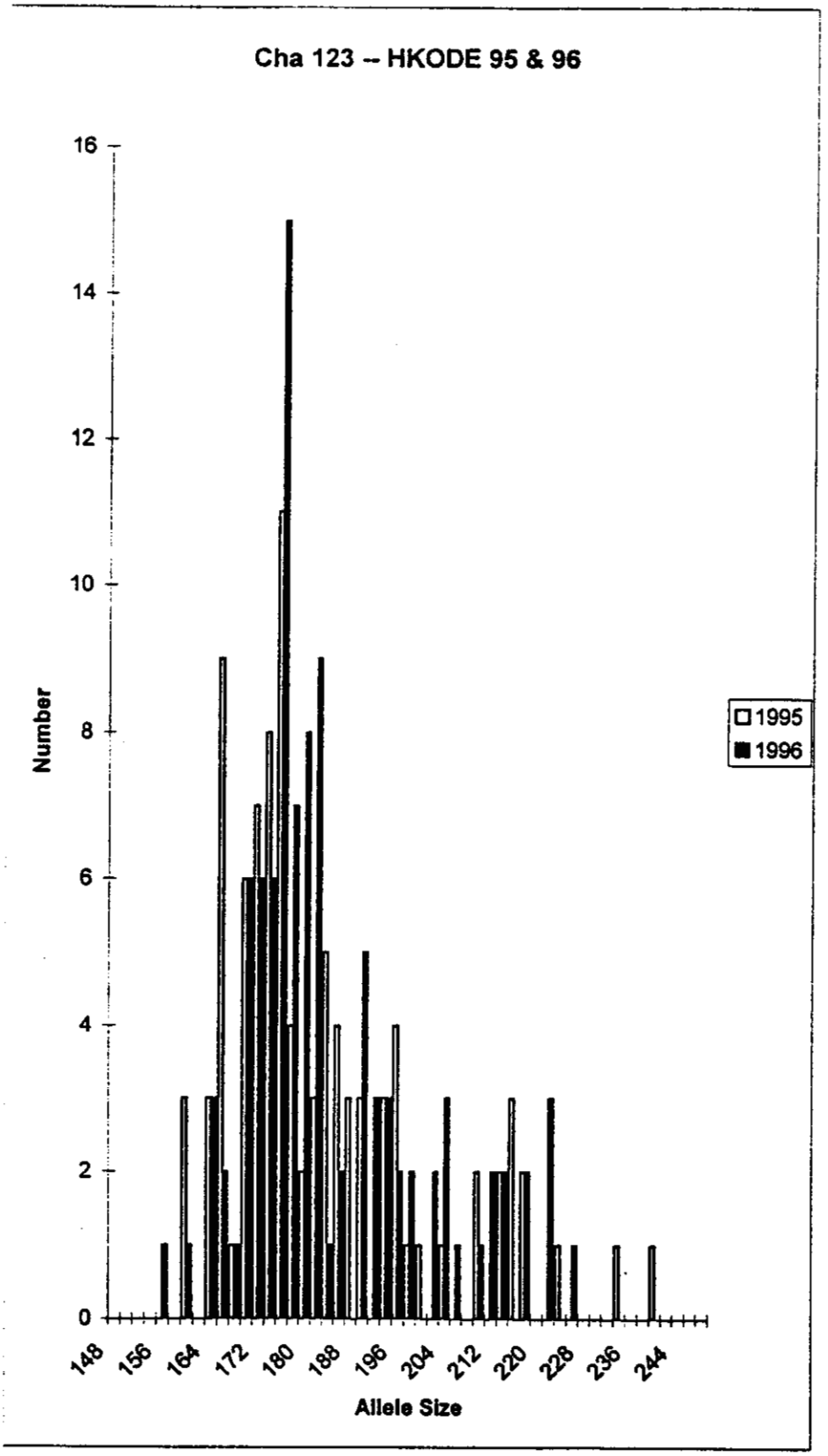

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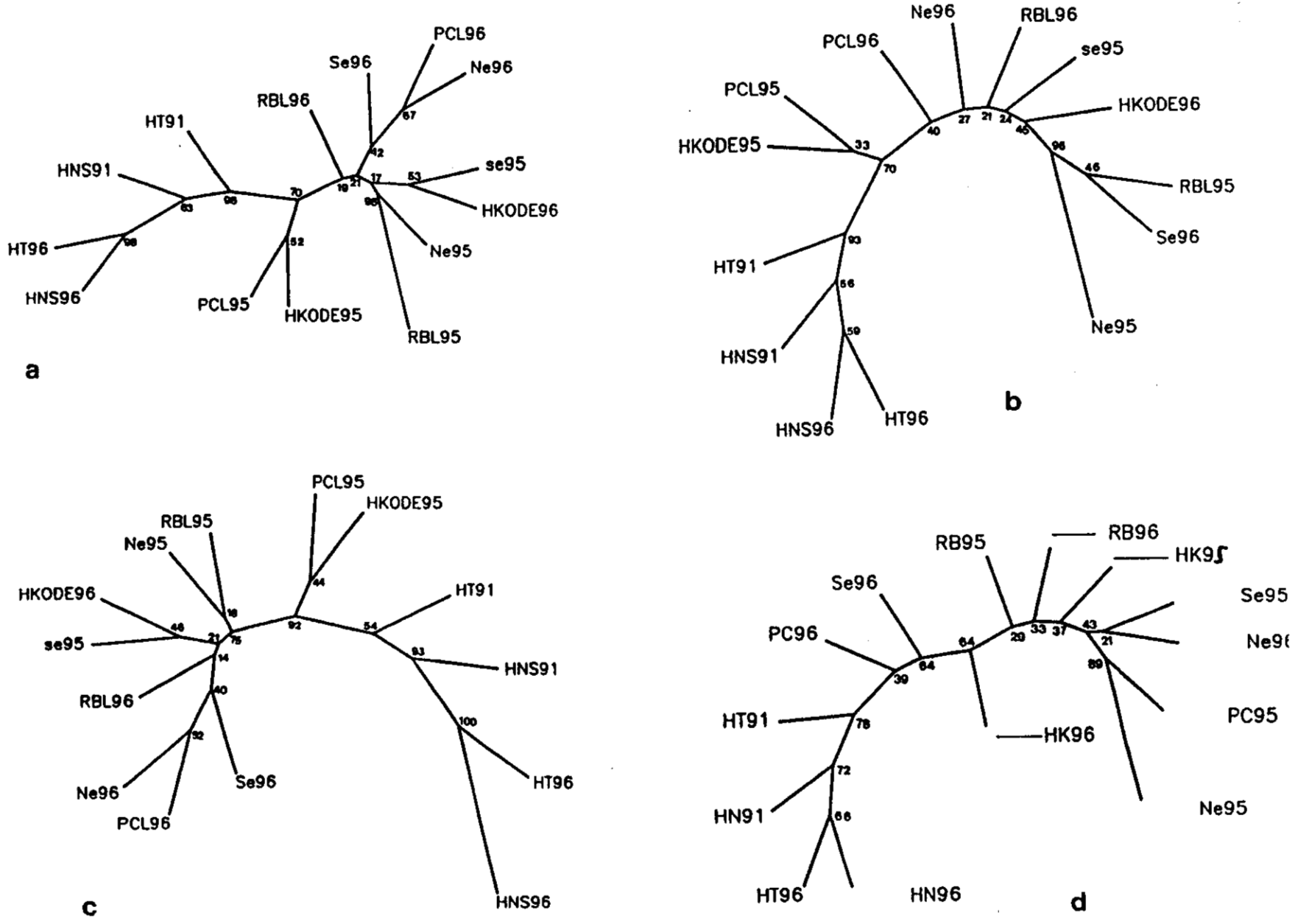

Figure 7. Genetic distance relationships for populations sampled over two years based on a) Cavalli-Sfortza distance; b) Reynold's distance; c) CONTML; and d) $\delta\mu^2$. Values on each node represent bootstrap values from 100 replicates.

APPENDIX D

TITLE: Development of Mitochondrial DNA Markers and Use to Screen Prince William Sound Herring Populations for Genetic Differentiation

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Commercial Fisheries Management & Development Division
Genetics Laboratory
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DATE: January 1997

Introduction

The goal of this project was to evaluate genetic differentiation of herring (*Clupea pallasi*) in Prince William Sound (PWS) using RFLP analysis of enzymatically amplified mitochondrial DNA (mtDNA). In preliminary experiments we identified a two kilobase (kb) segment of mtDNA corresponding largely to the ND1 gene as a promising target for study. We were able to achieve robust amplification of this mtDNA segment using the polymerase chain reaction (PCR), and preliminary tests with a variety of restriction enzymes revealed extensive polymorphism within this region of the herring mitochondrial genome.

Methods

Tissue samples were collected from seven Alaskan herring spawning sites (four within PWS and three outside; Fig. 1) by ADF&G personnel, and frozen prior to transfer into EtOH and shipment to the MMBL. DNA extractions were attempted for all 700 herring samples provided by ADF&G. For most samples, DNA was released from the tissue using a rapid lysis procedure (Olsen et al. 1996), but for some samples highly purified DNA was prepared using a conventional phenol-chloroform method (Hoelzel and Green 1992).

PCR primers used to amplify the ND1 gene were those designed by Cronin et al. (1993). Heterogeneity in haplotype frequencies among population samples was evaluated using the $\chi 2$ Monte Carlo approach described by Roff and Bentzen (1989) as implemented in the CHIRXC program (Zaykin and Pudovkin 199x).

Results and Discussion

Sample Sizes

Although PCR amplification and subsequent RFLP analysis was attempted at least once for each of the 700 samples, a substantial number of samples failed to produce interpretable data for one or more enzymes. In the majority of cases, such failures were attributable to weak or failed amplification of the target fragment, but in some cases anomalies associated with particular enzyme digests or electrophoretic gels led to results that could not readily be interpreted in terms of simple restriction site variation in the target sequence. The number of successfully scored samples varied among enzymes (N = 445-563; mean = 525; Table 1). Although samples that failed to give interpretable results with one enzyme often failed to do with others as well, the respective sets of "successful" and "failed" samples nonetheless differed substantially among enzymes; hence, sample sizes for successfully scored multi-enzyme haplotypes were further reduced. For this reason, I have analyzed and presented the data in terms of haplotype frequencies for single enzymes as well as haplotype frequencies two different two-enzyme composite data sets. Although I adopted this approach because of the sample size problem brought about by analysis failures, it was also convenient in light of another aspect of the herring mtDNA data: the high degree of polymorphism that we observed.

Haplotype Diversities

The four restriction enzymes used each detected 4-9 haplotypes (mean = 7; Table 1). Two two-enzyme combinations, *Ban*II+*Cfo*I and *Ban*II+*Rsa*I detected 22 and 26 haplotypes, respectively (Table 2). Among 367 individuals scored for all three highly variable enzymes, *Ban*II, *Cfo*I and *Rsa*I there were 40 composite haplotypes (Table 3). The full four-enzyme combination detected over 50 haplotypes among the herring successfully analyzed for this combination (data not shown). Single enzyme haplotype diversities ranged from a low of zero for *Rsa*I in Togiak Bay to 0.78 for *Ban*II in Norton Sound (Table 3). Mean single enzyme haplotype diversities ranged from 0.07 (*Hin*fI) to 0.65 (*Ban*II). Because of the low haplotype diversity detected by *Hin*fI and the fact that including it in analyses of multi-enzyme haplotype frequencies would have led to reduced sample sizes, I have not included results from this enzyme in multi-enzyme analyses.

Heterogeneity in haplotype frequencies among samples

Haplotype frequencies differed subtantially between Bering Sea and Gulf of Alaska samples. This was clearly evident for single and multi-enzyme haplotype frequencies involving each of the three highly variable enzymes (Tables1-3). The extreme differentiation between Bering and GOA samples is most evident for single enzyme haplotypes detected with *Rsa*I (Figure 1).

I used χ^2 pseudoprobabality contingency tests to evaluate heterogeneity in single and multi-enzyme haplotype frequencies among GOA samples (Table 5). I conducted two categories of tests: tests including and tests excluding Kodiak Island. Since all mitochondrial polymorphism is completely linked, tests involving different combinations of enzymes used on the same individuals should not be viewed as independent tests of statistical significance. Rather, they are meant as exploratory indicators of the heterogeneity observed with different enzyme combinations.

The results in Table 5 suggest that Kodiak Island herring may be genetically distinguishable from PWS herring. This inference is supported by low probabilities of haplotype frequency homogeneity for *Ban*II ($P = 0.009$), *Rsa*I ($P = 0.091$) and for *Ban*II/*Rsa*I composite data ($P = 0.039$). It is also supported by the observation that, except for the *Hin*fI data, all test probabilities for haplotype frequency homogeneity are lower for each single and multi-enzyme combination when Kodiak Island is included in the comparison (Table 5). The results of the contingency tests also suggest that there is little convincing evidence of significant heterogeneity in haplotype frequencies among the PWS samples. One test result (*Ban*II/*Rsa*I) did appear marginally "significant" (P = 0.048), but this result must be interpreted with caution for the reason of non-independence and multiple testing noted above.

Conclusions and current and future work

Two striking results have come out of this study so far. The first is the dramatic differentiation between GOA and Bering Sea herring (Figure 1). Although previous allozyme results indicating a "racial" split between western Pacific (including Bering Sea) and eastern Pacific (Grant 19xx) led us to expect significant differentiation of mtDNA between these regions, the extreme degree of mitochondrial differentiation that we observed was nonetheless a surprise. The second remarkable result was the high degree of genetic variability that we detected in the ND1 gene of Pacific herring. Although we surveyed a relatively short segment of herring mtDNA (2 kb) with only four restriction enzymes (one of which detected little variation), we nonetheless observed composite haplotype diversities of 90% or greater in a number of

populations. This result suggests that mtDNA haplotype diversity in Pacific herring may be comparable to that in the most variable marine teleost studied to date, another clupeid, Atlantic menhaden (species name; ref).

Apart from the very strong differentiation of Bering and GOA herring populations, our results suggest that Kodiak Island herring may be genetically distinguishable from PWS herring. This potential differentiation is most evident in the single-enzyme *Ban*II haplotype frequencies (Figure 2).

The large number of haplotypes that we observed in Pacific herring exacerbated another problem that we encountered, the failure of numerous samples to amplify and/or produce interpretable restriction digest results. The relatively high failure rate may be a result of the manner in which the samples were acquired and processed, since we detected evidence of DNA degradation in many samples. In addition to DNA degradation, samples that have deteriorated between the field and the laboratory may also have accumulated tissue degradation products that inhibit PCR. We have experienced greater difficulty amplifying both mtDNA and microsatellites from previously frozen samples than for fresh/EtOH preserved samples in several studies in this laboratory. Both DNA degradation and the accumulation of PCR inhibitors may pose greater problems for the amplification of the relatively large mtDNA fragment used in this study than for the much smaller microsatellites analyzed in the other part of this project. For this reason we have opted to do complete phenol-chloroform extractions on this year's samples, rather than the lysis procedure we used last year. We have also re-extracted (using phenol-chloroform) many samples from last year's collections, and will continue to do so to the extent that time and resources permit in an attempt to bolster last year's sample sizes. Because of the large number of haplotypes involved, we will also need to rerun many samples from both years on the same gels to ensure consistency of haplotype scoring.

References

Cronin, MA, Spearman, WJ, Wilmot, RL, Patton, JC, and Bickham, JW. 1993. Mitochondrial DNA variation in chinook (Oncorhynchus tshawytscha) and chum salmon (O. keta) detected by restriction enzyme analysis of polymerase chain reaction (PCR) products. Can. J. Fish. Aquat. Sci. 50:708-715.

Hoelzel, AR, and Green, A. 1992. Analysis of population level variation by sequencing PCR-amplified DNA. In: Molecular Genetic Analysis of Populations, A Practical Approach, Hoelzel, AR (ed.). Oxford University Press, New York.

Olsen, JB, Wenburg, JK, and Bentzen, P. 1996. Semiautomated multilocus genotyping of Pacific salmon (*Oncorhynchus* spp.) using microsatellites. Mol. Mar. Biol. Biotech. 5:259-272.

Roff, DA, and Bentzen, P. 1989. The statistical analysis of mitochondrial DNA polymorphisms: Chi-square and the problem of small samples. Mol. Biol. Evol. 6:539-545.

Zaykin, DV, and Pudovkin, AI. 1993. Two programs to estimate significance of $\chi 2$ values using pseudoprobability tests. J. Hered. 84:152.

Table 1. Frequencies of single enzyme mtDNA haplotypes in herring samples.

A. *Ban*II

	----------HAPLOTYPE----------								
LOCATION	A	B	C	D	E	F	G	H	**Total**
Norton Sound	21	25	18	0	2	0	0	0	**66**
Togiak Bay	23	24	26	0	4	3	0	0	**80**
Kodiak Island	40	34	8	1	2	3	0	0	**88**
St. Mathews Bay	30	35	2	0	2	1	0	0	**70**
Fish Bay	40	31	9	0	1	0	1	0	**82**
Pt. Chalmers	47	25	12	0	1	2	0	1	**88**
Rocky Bay	44	27	10	1	1	4	0	1	**88**
Total	**245**	**201**	**85**	**2**	**13**	**13**	**1**	**2**	**562**

B. *Cfo*I

	--------HAPLOTYPE-------							
LOCATION	A	B	C	D	E	F	G	**Total**
Norton Sound	5	2	8	0	0	0	0	**15**
Togiak Bay	20	5	51	0	0	0	0	**76**
Kodiak Island	17	0	0	4	0	0	0	**21**
St. Mathews Bay	65	1	3	3	0	0	0	**72**
Fish Bay	69	5	6	6	1	0	1	**88**
Pt. Chalmers	65	4	5	4	3	1	0	**82**
Rocky Bay	71	5	6	6	2	0	1	**91**
Total	**312**	**22**	**79**	**23**	**6**	**1**	**2**	**445**

Table 1. Continued

C. *Hin*fI

	HAPLOTYPE				
LOCATION	A	B	C	D	**Total**
Norton Sound	88	1	0	0	**89**
Togiak Bay	80	1	0	0	**81**
Kodiak Island	79	3	0	0	**82**
St. Mathews Bay	37	2	0	0	**39**
Fish Bay	88	2	0	0	**90**
Pt. Chalmers	84	4	1	0	**89**
Rocky Bay	87	4	0	2	**93**
Total	**543**	**17**	**1**	**2**	**563**

D. *Rsa*I

	----------HAPLOTYPE----------									
LOCATION	A	B	C	D	I	J	E	F	G	**Total**
Norton Sound	83	1	0	0	0	0	2	1	0	**87**
Togiak Bay	71	0	0	0	0	0	0	0	0	**71**
Kodiak Island	16	54	8	1	1	1	0	0	0	**81**
St. Mathews Bay	0	23	4	0	0	0	1	0	1	**29**
Fish Bay	8	65	10	0	0	1	0	0	0	**84**
Pt. Chalmers	7	62	15	0	1	0	1	0	0	**86**
Rocky Bay	12	66	11	0	0	0	2	0	0	**91**
Total	**197**	**271**	**48**	**1**	**2**	**2**	**6**	**1**	**1**	**529**

Table 2. Frequencies of two-enzyme composite mtDNA haplotypes in herring samples.

A. *Ban*II/*Cfo*I

	HAPLOTYPE																						
LOCATION	AA	AB	AC	AD	AE	AG	BA	BB	BC	BD	BE	BF	BG	CA	CC	DA	EA	EC	FA	FC	GA	HA	**TOTAL**
Norton Sound	1		2	0	0	0	4	2	0	0	0	0	0	0	6	0	0	0	0	0	0	0	**14**
Togiak Bay	2	0	19	0	0	0	13	5	2	0	0	0	0	0	26	0	2	2	2	1	0	0	**74**
Kodiak Island	8	0	0	3	0	0	3	0	0	1	0	0	0	2	0	1	0	0	1	0	0	0	**19**
St. Mathews Bay	22	1	3	2	0	0	32	0	0	1	0	0	0	2	0	0	2	0	1	0	0	0	**66**
Fish Bay	24	5	6	4	0	0	27	0	0	2	1	0	1	9	0	0	1	0	0	0	1	0	**81**
Pt. Chalmers	31	3	6	2	2	0	19	1	0	2	1	1	0	11	0	0	1	0	2	0	0	1	**83**
Rocky Bay	32	5	4	1	1	1	21	0	0	5	1	0	0	8	2	1	1	0	4	0	0	1	**88**
TOTAL	**120**	**14**	**40**	**12**	**3**	**1**	**119**	**8**	**2**	**11**	**3**	**1**	**1**	**32**	**34**	**2**	**7**	**2**	**10**	**1**	**1**	**2**	**425**

Table 2. Continued.

B. *Ban*II/*Rsa*I

	HAPLOTYPE																									
LOCATION	AA	AB	AC	AD	AE	AI	AJ	BA	BB	BC	BE	BG	BI	BJ	BK	CA	CB	CC	DA	DB	EA	EB	F	FB	HB	HC	**Total**
Norton Sound	17	1	0	0	0	0	0	24	0	0	0	0	0	0	0	18	0	0	0	0	1	0		0	0	0	**61**
Togiak Bay	24	0	0	0	0	0	0	20	0	0	0	0	0	0	0	26	0	0	0	0	2	0		0	0	0	**75**
Kodiak Island	5	29	0	1	0	1	1	8	17	0	0	0	0	0	2	2	1	0	1	0	0	2		2	0	0	**73**
St. Mathews Bay	0	9	0	0	1	0	0	0	9	0	0	1	0	0	1	0	0	2	0	0	0	1		0	0	0	**24**
Fish Bay	1	36	1	0	0	0	0	6	21	1	0	0	0	1	0	1	0	0	0	0	0	1		0	0	0	**69**
Pt. Chalmers	3	41	1	0	0	0	0	4	16	0	1	0	1	0	1	0	0	0	0	0	0	1		2	1	0	**72**
Rocky Bay	4	38	1	0	0	0	0	5	17	0	2	0	0	0	0	2	0	8	0	1	0	0		4	0	1	**83**
TOTAL	**54**	**154**	**3**	**1**	**1**	**1**	**1**	**67**	**80**	**1**	**3**	**1**	**1**	**1**	**4**	**49**	**1**	**10**	**1**	**1**	**3**	**5**		**8**	**1**	**1**	**457**

Table 3. Frequencies of BanII+CfoI+RsaI composite mtDNA haplotypes in herring samples.

	HAPLOTYPE																								
LOCATION	AAA	AAB	AAC	AAE	AAI	AAJ	ABB	ABC	ACA	ACB	ADB	AEB	AGB	BAA	BAB	BAE	BAI	BAK	BBA	BBB	BCA	BDB	BEC	BEJ	BFB
Norton Sound	1	0	0	0	0	0	0	0	2	0	0	0	0	4	0	0	0	0	2	0	0	0	0	0	0
Togiak Bay	2	0	0	0	0	0	0	0	19	0	0	0	0	12	0	0	0	0	4	0	2	0	0	0	0
Kodiak Island	0	6	0	0	1	1	0	0	0	0	3	0	0	2	4	0	0	2	0	0	0	1	0	0	0
St. Mathews Bay	0	5	0	0	0	0	0	0	0	0	0	0	0	0	7	0	0	1	0	0	0	0	0	0	0
Fish Bay	0	23	0	0	0	0	3	1	1	5	4	0	0	5	19	0	0	1	0	0	0	2	0	1	0
Pt. Chalmers	2	27	1	0	0	0	3	0	0	5	2	2	0	4	12	1	1	0	0	1	0	2	1	0	1
Rocky Bay	2	27	1	0	0	0	6	0	2	2	2	1	1	6	13	2	0	0	0	0	0	3	0	0	0
Total	**7**	**88**	**2**	**0**	**1**	**1**	**12**	**1**	**24**	**12**	**11**	**3**	**1**	**33**	**55**	**3**	**1**	**4**	**6**	**1**	**2**	**8**	**1**	**1**	**1**

	HAPLOTYPE															
LOCATION	BGA	CAA	CAC	CCA	DAA	DAB	EAB	EAF	ECA	FAA	FAB	FCA	GAC	HAB	HAC	**Total**
Norton Sound	0	0	0	6	0	0	0	0	0	0	0	0	0	0	0	**15**
Togiak Bay	0	0	0	26	0	0	0	0	2	2	0	1	0	0	0	**70**
Kodiak Island	0	0	2	0	1	0	2	0	0	0	1	0	0	0	0	**26**
St. Mathews Bay	0	0	2	0	0	0	1	0	0	0	0	0	0	0	0	**16**
Fish Bay	1	1	7	0	0	0	1	0	0	0	0	0	1	0	0	**76**
Pt. Chalmers	0	0	11	0	0	0	1	0	0	0	2	0	0	1	0	**80**
Rocky Bay	0	1	7	1	0	1	0	1	0	0	4	0	0	0	1	**84**
Total	**1**	**2**	**29**	**33**	**1**	**1**	**5**	**1**	**2**	**2**	**7**	**1**	**1**	**1**	**1**	**367**

Table 4. Single and two-enzyme haplotype diversities for herring populations.

LOCATION	*Ban*II	*Cfo*I	*Hin*fI	*Rsa*I	*Ban*II+ *Cfo*I	*Ban*II+ *Rsa*I
Norton Sound	0.78	0.59	0.02	0.09	0.69	0.76
Togiak Bay	0.72	0.48	0.02	0.00	0.77	0.81
Kodiak Island	0.63	0.31	0.07	0.05	0.75	0.93
St. Mathews Bay	0.56	0.18	0.10	0.35	0.65	0.85
Fish Bay	0.61	0.37	0.04	0.38	0.78	0.90
Pt. Chalmers	0.61	0.36	0.11	0.44	0.78	0.95
Rocky Bay	0.64	0.38	0.12	0.44	0.79	0.94
Mean	0.65	0.38	0.07	0.25	0.74	0.88

Table 5. Probability of homogeneity of haplotype frequencies among Gulf of Alaska herring populations for different restriction enzyme combinations.

Enzyme combination	PWS*	PWS+ Kodiak
*Ban*II	0.234	0.009
*Cfo*I	0.912	0.665
*Hin*fI	0.531	0.58
*Rsa*I	0.178	0.091
*Ban*II+*Cfo*I	0.36	0.287
*Ban*II+*Rsa*I	0.048	0.039
*Ban*II+*Rsa*I+*Cfo*I	0.849	0.52

* St. Mathews Bay, Fish Bay, Pt. Chalmers and Rocky Bay

APPENDIX E

Molecular Genetic Polymorphism in Alaskan Herring (*Clupea pallasi*) and its Implications for Population Structure

Final report submitted to-

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INTRODUCTION

This report examines data on genetic polymorphism in Pacific herring (*Clupea pallasi*) from the Bering Sea and Gulf Alaska gathered in two separate studies, one current and the other historical. The current study includes data reported here for the first time on mtDNA polymorphism in herring collected over two years from spawning aggregations in Prince William Sound, Kodiak Island, and the Bering Sea, as well as previously described data on microsatellite DNA polymorphism collected from the same specimens (Wright et al. 1996; Wright and Dillon 1997). The historical study comprises a comprehensive study of allozyme polymorphism in Pacific herring over its entire range, including a number of locations in the Bering Sea and Gulf of Alaska, and originally reported by Grant (1981) and Grant and Utter (1984). The primary goal of this report is to determine what data on molecular genetic variation in herring tell us about population structure of this species within Alaskan waters in general, and Prince William Sound in particular. Since interpretation of the biological significance of patterns of genetic variation is enhanced by incorporating information from as many genetic markers as possible, we have opted to conduct parallel analyses of the patterns of variation exhibited by the three types of genetic markers for which data exist on Alaskan herring: mtDNA, microsatellite, and allozyme. These comparative analyses form the focus of this document, rather than a detailed reiteration of the three data sets. Since (as noted above) the mtDNA data are new, they are accordingly described here in detail. Readers are referred to reports by the previously cited authors for similar details regarding the microsatellite and allozyme data.

METHODS

The locations of herring sampling sites are shown in Figure 1. DNA was extracted from tissue samples using two methods. DNA was released from most samples in the 1991/1995 collections (henceforth referred to as the "1995" samples) using a quick lysis procedure. Approximately 10 mg of tissue were added to 100 µL lysis buffer (40 mM Tris pH 9.5, 50 mM KCl, 0.5% Tween 20) along with 1 µL 10 mg • mL^{-1} proteinase K and incubated at 37 °C for 12 h, then heated to 95 °C for 15 min. Some 1995 samples and all 1996 samples were extracted to a high degree of purity using a phenol-chloroform extraction protocol. A quantity, 20-30 mg tissue, was added to 100 µL extraction buffer (10 mM Tris, pH 8.0, 50 mM NaCl, 2% SDS, 1 mM EDTA) along with 10 µL 10 mg • mL^{-1} proteinase K and 4 µL DTT and incubated overnight at 65 °C. The sample was then extracted once with phenol, once with phenol/chloroform, and once with chloroform. Following the organic extractions, 10 µL 5 M NaCl and 2 volumes of 100% EtOH were added, and the sample was incubated at –20 °C for at least 1 h, then centrifuged at 14,000 rpm in a bench-top microcentrifuge for 30 min. The supernatant was removed and the sample washed once with 1 mL 70% EtOH. The DNA was dried under vacuum, then resuspended in 100 µL TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA).

A 2 kilobase (kb) mtDNA fragment comprising the ND1 gene, all or part of three tRNA genes, and part of the 16S rRNA gene was amplified following Cronin et al. (1993). All 1995 samples, and some 1996 samples were amplified using the primers originally described in Cronin et al. In 1996 the primers were redesigned based on herring mtDNA sequence data (Bentzen unpublished data) in an effort to obtain more consistent amplification success. The redesigned primers were as follows: 16S, GCCTCGCCTGTTTACCAAAAACAT; ND1R, CGGGGTATGGGCCCGAAAGCTT. The 16S primer is a slightly modified version of the 16S

rRNA primer originally described by Kocher et al. (1989). The ND1R primer resides in a tRNA gene flanking ND1.

Most samples were amplified using Pharmacia Ready-to-Go PCR beads. These provide *Taq* polymerase, PCR buffer reagents, and dNTPs in dried form, and provided the most consistent amplification of the 2 kb ND1 fragment. Reactions contained 1 µL DNA template, 1.2 µL of each primer (10 µM) and 21.6 µL H_2O plus a PCR bead to give a total volume of 25 µL. PCR profiles were as follows: 1 cycle (95 °C, 5 min.); 35 cycles (95 °C , 1 min.; 55 °C, 1 min.; 72 °C, 2 min.).

Aliquots (usually comprising 5 µL) of each PCR product were digested in 10 µL volumes at 37 °C overnight with the restriction enzymes *Ban*II, *Rsa*I, and *Hha*I (or its isoschizomer, *Cfo*I) containing the appropriate 1× restriction buffer supplied by the manufacturer (Promega or New England Biolabs). A volume of the digest, usually 10 µL, was mixed with loading dye and subjected to electrophoresis in a 2% agarose/TBE gel at ~5.5 $V \bullet cm^{-1}$ for approximately 1.5 h. Each gel contained DNA size standards in at least 2 lanes. Gels were stained with SYBR Green (Molecular Probes) then scanned on a Molecular Dynamics FluorImager 575.

DNA fragments in some gels were sized using Molecular Dynamics FragmeNT Analysis software. MtDNA haplotypes revealed by each enzyme were assigned letter codes based on their order of detection. The likely mutational relationships among single-enzyme haplotypes were inferred under the assumption that all changes in fragment profiles were the result of gain or loss of restriction sites. The latter assumption was supported by the fact that fragment patterns for all haplotypes summed to just under 2 kb; hence, there was no evidence of insertions or deletions in any amplified fragments.

Probability tests of heterogeneity in genotype frequencies among samples, and tests of isolation by distance for mtDNA, microsatellites and allozymes were conducted using GENEPOP V3.1b (Raymond and Rousset 1995). Geographic distances between samples were estimated as the shortest marine path between sites. AMOVAs on all three types of genetic markers were conducted using ARLEQUIN V1.1 (Schneider et al. 1997).

RESULTS

mtDNA data

We obtained complete (3-enzyme) genotypic data from 880 out of a total of 1,400 herring that we attempted to analyze. The remainder either yielded incomplete data (App. I) or none at all, despite efforts to re-extract and re-amplify these samples. The number of individuals that yielded complete data varied substantially among samples collected in different locations and years (App. II, Table 1), suggesting that differences in the quality of tissue preservation among different collections of herring may have contributed to our varying success rate. This possibility is further suggested by the fact that the number of samples for which we were able to obtain complete data in 1996 (390) declined from 1995 (490), despite the fact that we improved our method of DNA extraction, redesigned the PCR primers and improved our PCR protocol in 1996.

Polymorphism in the herring mtDNA detected by the three restriction enzymes was extensive. The enzymes *Ban*II, *Hha*I, and *Rsa*I revealed 8, 9 and 11 single-enzyme haplotypes, respectively (Table 2, Figure 2). Differences in the fragment patterns among single locus

haplotypes could all be readily interpreted in terms of the gain or loss of single restriction sites (Figure 3). Taken in combination, the three enzymes revealed 52 composite haplotypes among the herring, with an average haplotype diversity (h) among samples of 0.805 (Table 1).

The frequencies of common haplotypes varied greatly between Bering Sea and Gulf of Alaska samples (App. III, Figure 4). For instance, for the haplotypes with the highest overall frequency in the Gulf of Alaska, AAB (34%) and BAB (18%), the corresponding frequencies in the Bering Sea were 0.6% and 0%, respectively. Similarly, the three most common Bering Sea haplotypes, BAA (23%), CCA (29%) and ACA (28%) accounted for 6.5%, 0.8% and 1.1% of Gulf of Alaska samples, respectively.

Figure 5 shows hypothesized mutational relationships of the composite haplotypes of herring mtDNA along with their relative frequencies in the Gulf of Alaska and Bering Sea. Several points are evident from this figure. First, it appears that the Gulf of Alaska and Bering Sea are each dominated by one or two clusters of mitochondrial genotypes. These clusters may constitute distinct clades, although this interpretation is tentative because of the limited genetic information available from only three restriction enzymes. Second, the clusters show relatively little overlap in distribution between the two sea basins. The degree of overlap varies among clusters. The 'AAB, BAB, ACB, CAC' cluster is almost exclusively limited to the Gulf of Alaska; the 'ACA, CCA' cluster is primarily present in the Bering Sea and rare in the Gulf of Alaska; finally, the 'AAA, BAA, BBA' cluster is mostly represented by Bering Sea herring but is also widespread at low frequencies in the Gulf of Alaska. Third, the hypothesized 'Gulf of Alaska' cluster(s) include many more rare derivative haplotypes than seen in the 'Bering Sea' cluster(s).

Heterogeneity in the frequencies of haplotypes was also significant among all samples collected in the Gulf of Alaska ($P = 0.003$) as well as among all samples collected in Prince William Sound ($P = 0.036$), but not among all samples collected from the Bering Sea ($P = 0.143$) (Table 3). Within particular sampling years, heterogeneity in haplotype frequencies was significant for Prince William Sound in 1996 ($P = 0.012$), but for not for any other comparison (Table 3).

Comparative analysis of mtDNA, microsatellite and allozyme data.

Analysis of Molecular Variance (AMOVA):

All three classes of marker, mtDNA (described above), microsatellites (Wright et al. 1996; Wright and Dillon 1997) and allozyme (Grant 1981; Grant and Utter 1984) revealed substantial genetic differentiation between Bering Sea and Gulf of Alaska populations. To compare the relative magnitudes of genetic variation among and within the two sea basins across the three classes of genetic marker, we conducted AMOVAs on the three data sets (Tables 4). As expected, all three marker classes showed highly significant differentiation among the two sea basins ($P < 0.001$); however, the three marker types varied substantially in the absolute magnitude of the among basins differentiation revealed. The among basins proportion of genetic variance (F_{BT}) was 0.241 for allozymes and 0.169 for mtDNA, but only 0.023 for microsatellites. However, when the microsatellite AMOVA was conducting taking mutational distance among alleles into account, the F_{BT} component for these markers increased to 0.209. The within basins proportion of genetic variance (F_{SB}) showed a different pattern among markers. F_{SB} for allozymes (0.003) was not significant ($P = 0.488$), whereas it was greater (0.013-0.03) and significant ($P < 0.001$) for mtDNA and microsatellites.

To further assess the significant within basin component (F_{SB}) of genetic variance detected with microsatellites and mtDNA, we conducted additional AMOVAs focusing on samples collected in 1995 and 1996 within the Gulf of Alaska and analyzed with these markers. In this case we compared the relative proportions of genetic variance attributable to spatial and temporal factors. In one AMOVA we grouped GOA samples by location (Table 5A). This analysis revealed that the proportion of genetic variance that occurred among samples (years) within locations was similar for both microsatellites ($F_{SL} = 0.011$, $P < 0.001$) and mtDNA ($F_{SL} = 0.012$, $P = 0.445$). By contrast, estimates of the proportion of genetic variance associated with variation among locations after removing the temporal effect were actually negative for both markers ($F_{LT} = -0.003$) and of course, not significant ($P \geq 0.88$).

In a second AMOVA conducted on GOA samples we grouped the samples by year (Table 5B). In this case the proportion of genetic variance among years was the same ($F_{YT} = 0.003$) and significant or marginally significant for both marker types ($P = 0.029$ and $P = 0.054$ for microsatellites and mtDNA). The proportion of genetic variance among all GOA samples within years (F_{SY}) in this analysis was 0.008 for microsatellites and significant ($P < 0.008$); whereas, for mtDNA F_{SY} was similar (0.008) but not significant ($P = 0.226$).

Tests of isolation by distance:

We tested whether genetic distance and geographically distance were significantly correlated for each of the three genetic marker classes. When samples collected from the Bering Sea and Gulf of Alaska were considered together, all three types of genetic marker revealed a highly significant relationship between genetic distance and geographic distance (Mantel test, $P < 0.001$). However, it is evident from Figure 6 that for mtDNA and allozymes, at least, the correlation between genetic and geographic distances is driven largely by the genetic differentiation between the Bering Sea and Gulf of Alaska. This is also evident for microsatellites (Figure 6A), although less clearly so than for the other markers. The lesser magnitude of genetic differentiation among basins exhibited by microsatellites relative to mtDNA and allozymes is also evident from comparison of panels A, B and C in Figure 6.

DISCUSSION

Our results suggest a complex picture of genetic variation within and among herring populations that must be viewed on two spatial scales: Gulf of Alaska versus Bering Sea, and within each of these sea basins. Our results also have implications for the types of information yielded by different classes of molecular markers, and how genetic research on north Pacific/Bering Sea herring might best proceed in future. These issues are considered below.

Gulf of Alaska versus Bering Sea:

Our results confirm and extend conclusions reached earlier by Grant (1981) and Grant and Utter (1984) that Bering Sea and Gulf of Alaska herring are genetically distinct. Those authors considered herring in the two sea basins to represent separate "races" of herring. Notwithstanding the vagueness of the term, our mtDNA data support this interpretation. The dominant mitochondrial haplotypes in the two sea basins are different; moreover, these differing haplotypes fall into larger haplotype clusters which may represent distinct clades that are themselves primarily restricted to individual sea basins (Figure 5). Interpretation of the haplotype

clusters as clades is tentative, because of the limited resolution afforded by the use of only three restriction enzymes, as well as evidence of homoplasy (parallel gain or loss of restriction sites) evident from inspection of Figure 5. Nonetheless, it is clear from both the mtDNA data and the allozyme data that there is very little mixing of herring between the two regions. Using the 'F_{ST}' method of estimating gene flow (Wright 1951) and the F_{BT} estimates in Table 4, the migration rate between the two basins can be estimated to be 0.8 fish per generation on the basis of the allozyme data, and 2.4 females per generation on the basis of the mtDNA data. Even these relatively low values may be overestimates, since this method assumes that alleles/haplotypes are shared on the basis of contemporary gene flow. However, the non-random pattern of "clade" sharing between the Bering Sea and Gulf of Alaska seen in Figure 5 suggests that this assumption is not valid, at least for mtDNA haplotypes, and that the sharing of haplotypes between the Bering Sea and Gulf of Alaska is more a consequence of historical effects than contemporary gene flow.

The 'among basins' differentiation observed with microsatellites (F_{BT} = 0.023; Table 4) was an order of magnitude less than that seen with allozymes (F_{BT} = 0.241). Since migration and drift are expected to have a similar effect on all neutral nuclear markers, the large difference in the extent of differentiation exhibited by the two classes of genetic marker must be attributable to differential effects of natural selection, or mutation, or some combination of the two forces. There is evidence that suggests that some of the allozyme loci may be influenced by selection (Bentzen, unpublished data). However, it is likely that mutations in the rapidly evolving microsatellite loci have also directly or indirectly promoted the low apparent divergence of microsatellite loci between the two sea basin assemblages of herring. The indirect effect is a simple consequence of the high heterozygosity of the microsatellite loci, which averaged close to 90% in most populations (Figure 7). Since F_{ST} (F_{BT}) measures the proportional reduction in heterozygosity due to population subdivision, the high 'local' heterozygosities of the microsatellite loci establishes an upper bound on measurable F_{ST} values with these loci of ~0.1, well below the apparent 'true' value of this parameter estimated with allozymes and mtDNA. The potential direct effect of mutation in reducing microsatellite differentiation between the two sea basins may be a consequence of the high mutation rates and step mutational processes believed to predominate at microsatellite loci (reviewed in O'Connell and Wright 1997). In this 'mutational environment' many of the microsatellite alleles that appear to be shared in the two sea basins may be the result of homoplasic mutations. In this scenario, at least some alleles are shared in state, but not by descent across the Bering-Gulf of Alaska boundary. Some circumstantial evidence in support of this scenario comes from the fact that when we conducted the microsatellite AMOVA incorporating estimates of mutational distance between alleles (based on the step mutation model), the microsatellite-derived estimate of F_{BT} (0.209; Table 4) was comparable to the values obtained with much slower mutating mtDNA and allozymes under a purely drift/migration-based model. A more definitive test of this homoplasy scenario will require sequencing of microsatellite alleles from both major groups of herring.

Within-basin genetic variation:

The three classes of genetic markers all revealed less genetic variation within the two sea basins than among them (Table 4). Apart from this broad level of congruence, there were differences in the levels of statistical significance associated with variation revealed by the different types of markers. For microsatellites, allelic frequency variation among samples collected in both years was significant on all spatial scales including within the Bering Sea,

within the Gulf of Alaska, and within Prince William Sound (Wright et al. 1996; Wright and Dillon 1997; Table 4 this report). By contrast, mtDNA haplotype frequency variation was not significant among any samples collected in the Bering Sea, or among samples collected in the Gulf of Alaska in 1995 (Table 3). Variation in mtDNA haplotype frequencies was significant among samples collected in Prince William Sound in 1996 (Table 3). Grant and Utter (1984) found significant allozyme differences between northern and southern Bering Sea populations, as well as between widespread populations in the Gulf of Alaska. These results are supported by additional statistical analyses (Bentzen, unpublished data), although not by the non-significant within-basin variance component (F_{SB}) AMOVA result for allozymes in Table 4.

For the DNA markers examined in this study, comparisons among samples taken in the same locations but in different years yielded an important result: the magnitude of genetic variation among sampling years within locations was equal to or greater than the magnitude of variation among locations within sea basins (Table 5). Thus, even though the microsatellites revealed significant differences among population samples on geographic scales as fine as within Prince William Sound, these differences were not reproducible from year to year. In general, samples drawn from the same locations in different years did not group together in dendrograms based on genetic distance (see Figure 7, Wright and Dillon 1997). Even when all samples collected within the Gulf of Alaska were pooled by year, variation among years was significant for microsatellites ($P = 0.029$) and only marginally insignificant for mtDNA ($P = 0.054$) (Table 5B).

Conclusions and suggestions for further research:

The allozyme, microsatellite and mtDNA data clearly indicate that there is a marked genetic discontinuity between herring in the Bering Sea and Gulf of Alaska. Genetic discontinuities of this sort are uncommon in continuously distributed pelagic marine species, but a number of similar examples have been encountered, particularly around the Florida peninsula and other known zoogeographic boundaries (reviewed in Shaklee and Bentzen 1998). By contrast, the same data indicate that differentiation within these sea basins is of much lesser magnitude. Moreover, the DNA data provide no evidence of stable differentiation among populations within sea basins on spatial scales of up to ~700 km. Rather, the DNA data suggest that temporal variation among spawning aggregations dominates genetic variability on these spatial scales. Such variation has been observed in a number of other pelagic marine fishes, including California sardine (*Sardinops sagax caeruleus*) and northern anchovy (*Engraulis mordax*) (Hedgecock 1994) as well as various pelagic marine invertebrates (reviewed in Shaklee and Bentzen 1998) and has been termed 'chaotic patchiness' (Hedgecock 1994). The processes that produce chaotic patchiness are not entirely clear, but Hedgecock (1994) has suggested that it may result largely from genetic drift brought about by 'sweepstakes' reproductive success in which relatively few individuals participating in any given spawning effort produce recruits for the next generation.

The lack of stable differences in allele frequencies among herring spawning aggregations do not disprove the existence of demographically independent local spawning stocks, but nor do they provide any positive evidence in support of this possibility. Analyses of isolation by distance suggest that detectable genetic differentiation may occur among herring populations (of the same race) separated by ~1,000 km or more (Bentzen unpublished data). However, it seems unlikely that further work with neutral DNA markers will firmly resolve the question of whether demographically independent stocks occur within Prince William Sound or even in the northern

Gulf of Alaska. Grant and Utter (1984) did find evidence of genetic differentiation within both the Bering Sea and across the Gulf of Alaska with allozyme markers, and although they did sample some locations in two different years they did not report any evidence of strong temporal variation. Whether these apparent qualitative differences between the results obtained with protein and DNA markers merely reflect sampling artifacts, or whether some feature of the allozymes (perhaps selection) make them more temporally stable, is unclear. Regardless, further allozyme work on this species might be warranted.

Further work with DNA markers is also warranted, for at least two reasons. First, the striking differentiation between the Bering Sea and Gulf of Alaska, and the exact position and the nature of the boundary between these two genetic races deserves further investigation. Second, different sampling protocols might yield useful insights into the processes driving genetic differentiation on small—medium geographic scales. One useful experiment would examine replicate samples drawn from particular spawning areas over short periods of time (days--weeks?) to probe the finest scales of (temporal) genetic differentiation. Additionally, samples drawn from different (spatially defined) spawning aggregations within a region (such as Prince William Sound) but stratified according to the age of the fish could be used to dissect the importance of variation among year classes relative to local (spatially driven) differentiation.

Further work with DNA markers might involve microsatellites or mtDNA, or both—however, it would be worth considering adopting new versions of these markers. The ND1 fragment used in this study proved to be difficult to amplify in many samples. Since herring mtDNA exhibits a high level of genetic variation, it should be possible to identify somewhat shorter, more robustly amplifiable mtDNA fragments from this species that would exhibit adequate genetic variation for further assay. Similarly, the microsatellites used thus far, although robust in amplification, exhibit sub-optimally high levels of genetic variation, as well as difficulties with interpretation brought about by PCR "stutter". A further troubling feature of the current suite of dinucleotide microsatellites is the frequent occurrence of significant heterozygote deficiencies with these loci (see Table 2, Wright et al. 1996; Table 2 Wright and Dillon 1997). These heterozygote deficiencies might result from Wahlund effects associated with the temporal variability/chaotic patchiness, but they may also result from null alleles, or other genotype scoring difficulties. Adoption of less variable, more easily scorable tetranucleotide microsatellites might alleviate these problems.

REFERENCES

Cronin, M.A., Spearman, W.J., and Wilmot, R.L. 1993. Mitochondrial DNA variation in chinook (*Oncorhynchus tshawytscha*) and chum salmon (*O. keta*) detected by restriction enzyme analysis of polymerase chain reaction (PCR) products. Can. J. Fish. Aquat. Sci. 50:708-715.

Grant, W.S. 1981. Biochemical genetic variation, population structure, and evolution of Atlantic and Pacific herring. Ph.D. dissertation, University of Washington, Seattle, WA. 133 p.

Grant, W.S., and Utter, F.M. 1984. Biochemical population genetics of Pacific herring (*Clupea pallasi*). Can. J. Fish. Aquat. Sci. 41:856-864.

Hedgecock, D. 1994. Temporal and spatial genetic structure of marine animal populations in the California current. CalCOFI Rep. 35:73-81.

Kocher, T.D., Thomas, W.K., Meyer, A., Edwards, S.V., Pääbo, S., Villablanca, F.X., and Wilson, A.C. 1989. Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. Proc. Natl. Acad. Sci. USA 86:6196-6200.

O'Connell, M., and Wright, J.M. 1997. Microsatellite DNA in fishes. Rev. Fish Biol. Fish.:7:331-363.

Raymond, M. and Rousset, F. 1995. GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. J. Heredity: 86:248-249.

Rousset, F. 1997. Genetic differentiation and estimation of gene flow from *F*-statistics under isolation by distance. Genetics 145:1219-1228.

Schneider, S., Kueffer, J.-M., Roessli, D., and Excoffier, L. 1997. ARLEQUIN ver 1.1. A software for population genetic data analysis. Genetics and Biometry Laboratory, Dept. of Anthropology and Ecology, University of Geneva, CP 511, 1211 Geneva 24, Switzerland.

Shaklee, J.B., and Bentzen, P. 1998. Genetic identification of stocks of marine fish and shellfish. Bull. Mar. Sci. (in press)

Wright, J.M., and Dillon, M.C. 1997. Temporal stability of microsatellite markers in Prince William Sound herring populations. Report submitted to Alaska Dept. of Fish and Game.

Wright, J.M., Dillon, M.C., and O'Connell, M. 1996. Development of microsatellite markers for genetic discrimination of Prince William Sound herring populations. eport submitted to Alaska Dept. of Fish and Game.

Wright, S. 1951. The genetical structure of populations. Ann. Eugen. 15:323-354.

Table 1. Summary statistics for herring mtDNA samples.

Sample	***N***	***A***	***h***
Norton 91	15	5	0.729
Norton 96	23	6	0.715
Togiak 91	71	9	0.754
Togiak 96	48	9	0.779
Kodiak 95	70	18	0.847
Kodiak 96	85	18	0.795
Pt. Chalmers 95	84	21	0.844
Pt. Chalmers 96	86	16	0.793
Rocky Bay 95	87	22	0.844
Rocky Bay 96	11	7	0.744
St. Mathews 95	87	16	0.802
St. Mathews 96	53	10	0.774
Fish Bay 95	76	16	0.823
Fish Bay 96	84	21	0.860
Bering Sea	157	12	0.778
Gulf of Alaska	723	47	0.833
Total	**880**	**52**	
Mean			**0.805**

N, number of individuals that yielded 3-enzyme data
A, number of haplotypes
h, haplotype diversity

Table 2. Fragment sizes observed in herring mtDNA haplotypes.

***Ban*II**	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**
	1130	1130			1130	1130		
			975				975	
								830
		790		790			790	
				790				
					700			
	395		395			395		395
	395		***395***					***395***
				360				
						300		300
			150				150	
						95		
					90			
TOTAL	**1920**	**1920**	**1915**	**1940**	**1920**	**1920**	**1915**	**1920**

***Hha*I/*Cfo*I**	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**
								1570
	920					920		
		730						
	645	645	645	645	645	645	645	
			645					
					570		570	
				495				
				430				
	380	380	380	380	380		380	380
					350			
			265					
						230		
							200	
						150	150	
		200						
TOTAL	**1945**	**1955**	**1935**	**1950**	**1945**	**1945**	**1945**	**1950**

Table 2. *(continued)*

***Rsa*I**

	A	B	C	D	E	F	G	H	I	J	K
									900		
						765					
	725	725		725			725				
	675	675	675	675	675	675	675	675	675	675	675
				585							
											550
			525								
					480						
										470	
							400				
	375					375					
								350			
										260	
					240						
	210	210	210		210	210		210	210	210	210
									210		
		190	190					190			190
			190								
		165	165				165	165			165
TOTAL	**1985**	**1965**	**1955**	**1985**	**1605**	**2025**	**1965**	**1590**	**1995**	**1615**	**1790**

Numbers in bold italics represent fragments that were not clearly resolved as separate fragments, but whose presence was inferred from stoichiometric considerations.

Table 3. Probability of homogeneity of mtDNA genotype frequencies among herring samples.

Test	*P*
all Bering Sea samples	0.1430
all Gulf of Alaska samples	0.0033
all Gulf of Alaska samples, 1995	0.3523
all Gulf of Alaska samples, 1996	0.0582
all Prince William Sound samples	0.0355
Prince William Sound, 1995	0.4425
Prince William Sound, 1996	0.0119

Table 4. AMOVA results, among and within sea basins. Shown are hierarchical F components and associated probabilities for the null hypothesis, F not greater than 0.

	microsatellite	**mtDNA**	**allozyme**	**microsat. (ρ)***
***F* component (*P*)**				
F_{SB}	0.010 (0.000)	0.013 (0.000)	0.003 (0.488)	0.030 (0.000)
F_{BT}	0.023 (0.000)	0.169 (0.000)	0.241 (0.000)	0.209 (0.000)
F_{ST}	0.033 (0.000)	0.178 (0.000)	0.244 (0.000)	0.233 (0.000)

* Allelic relationships inferred under step mutation model are incorporated in this AMOVA
F_{SB} = among samples within sea basins
F_{BT} = among sea basins
F_{ST} = among all samples

Table 5. AMOVA results for samples within Gulf of Alaska. Shown are hierarchical F components and associated probabilities for the null hypothesis, F not greater than 0.

A. Samples grouped by location.

F component (*P*)	microsatellite	mtDNA
F_{SL}	0.011 (0.000)	0.012 (0.445)
F_{LT}	-0.003 (0.978)	-0.003 (0.880)
F_{ST}	0.008 (0.000)	0.009 (0.203)

F_{SL} = among samples (years) within locations
F_{LT} = among locations
F_{ST} = among all samples

B. Samples grouped by year.

F component (*P*)	microsatellite	mtDNA
F_{SY}	0.008 (0.008)	0.008 (0.226)
F_{YT}	0.003 (0.029)	0.003 (0.054)
F_{ST}	0.010 (0.000)	0.010 (0.191)

F_{SY} = among samples within years
F_{YT} = among years
F_{ST} = among all samples

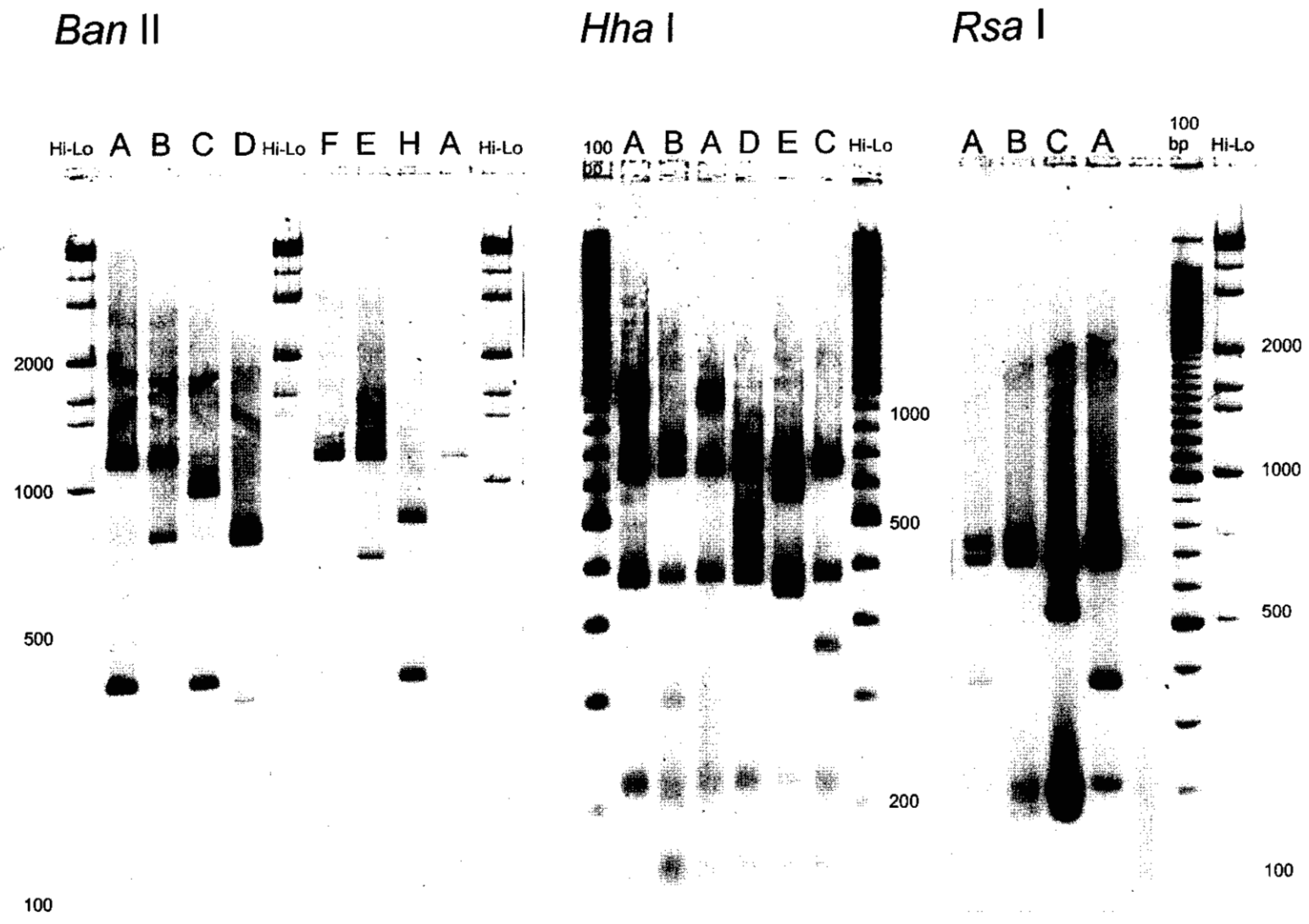

Figure 2. Common herring mtDNA haplotypes.

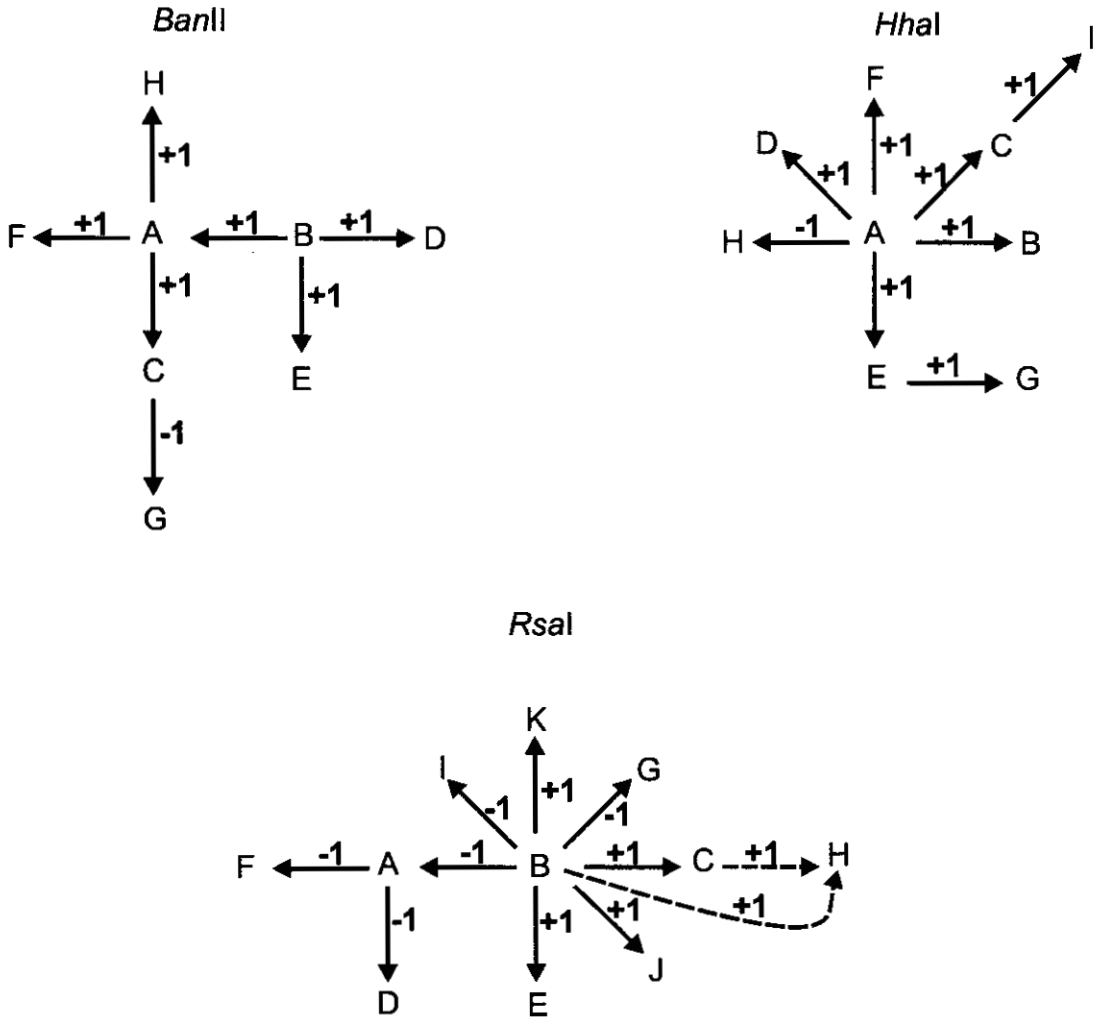

Figure 3. Hypothesized relationships of single-enzyme herring mtDNA haplotypes. Shown are the gain or loss of restriction sites that have occurred among haplotypes. Dashed lines indicate ambiguity in the identity of the most likely precursor to a given haplotype.

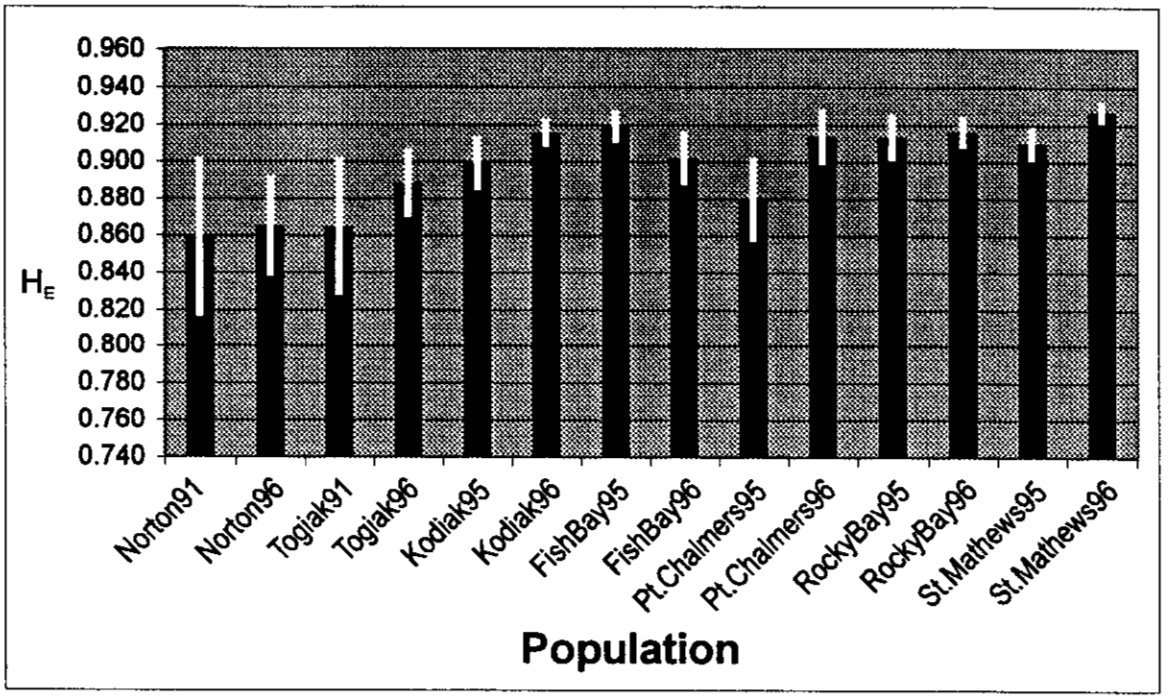

Figure 7. Mean expected heterozygosities of five herring microsatellite loci (+& SE). The mean number of alleles per locus was 34.