CYTOGENETIC STUDIES IN THREE SPECIES OF LARIDS AND THREE SPECIES OF ALCIDS

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CYTOGENETIC STUDIES IN THREE SPECIES OF LARIDS AND THREE SPECIES OF ALCIDS

BY

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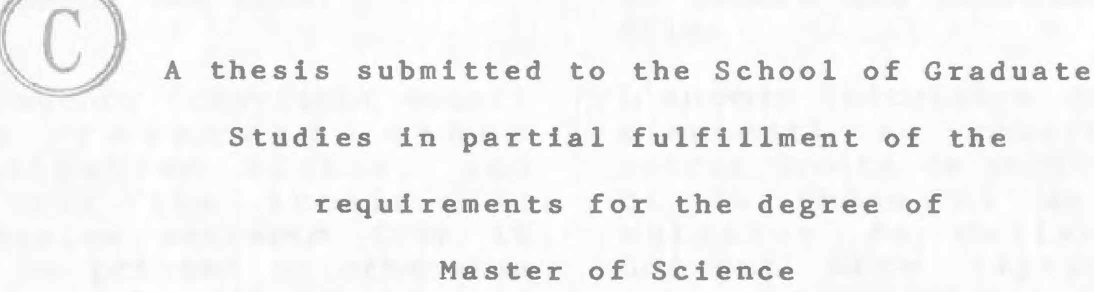

A thesis submitted to the School of Graduate Studies in partial fulfillment of the requirements for the degree of Master of Science

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December, 1985

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ISBN 0-315-36961-2

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ABSTRACT

The embryonic tissues of 3 larids (Larus argentatus, Larus marinus, Rissa tridactyla) and 3 species of alcids (Uria aalge, Alca torda, Fraterula arctica) were grown in vitro as primary explants to develop a method for harvesting mitotic chromosomes. A reliable method was developed, with chromosomes that were harvested being stained with a fluorescent dye. Partial karyotypes and idiograms of the largest 13 autosomes were made for each species. Based on the p arm to q arm ratios the centromere position for each chromosome was determined. Comparisons were then made between larid species and alcid species as well as between groups (larid/alcid) and the significant differences were noted.

ACKNOWLEDGEMENTS

I would like to express my appreciation to the following individuals for their contribution in making this study possible:

To Dr. William Threlfall, my supervisor, for suggesting this topic and supporting me financially for one summer and for his patience in helping to correct and edit this thesis.

To Dr. Gordon Bennett and Dr. George Moskovits for their help in editing this thesis.

To Dr. Penny Allderdice for the use of her laboratory equipment.

To Ms. Brenda Eales for her technical advice.

To Ms. Dub Porter, Ms. Ruth McLagan and Mr. Chuck Bourgeois for their assistance in collecting the seabird eggs.

To Mr. Roy Ficken, Department of Biology Photographer for his assistance with the photographs.

To Dr. Chris Ford, work supervisor, for his continued encouragement to the author to complete this thesis and for permitting the author to rearrange her work schedule in order to work on this project.

To Mr. John Bartlett for his patience and help.

To Mrs. Gen Wight for assisting in typing the Tables and Appendices.

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The term karyotype refers to the chromosome complement as regards to both number and morphology as it appears at mitotic metaphase. The chromosomes are arranged in pairs and lined up, starting with the largest and continuing in order of diminishing size. By convention, the shorter chromosome arm points towards the top of the page (Brown, 1972; Therman, 1980). An idiogram or karyogram is a diagrammatic karyotype based on chromosome measurements from many cells (Boyes et al., 1971; Brown, 1972). The concept of karyotyping was first used by Lewitsky (1931) for plant material.

Bird karyotypes are characterized as having a high diploid number of chromosomes ranging from 60-80 (Ray-Chaudhuri, 1973) compared with the human modal number of 46 and the 6 usually encountered in many Diptera. These chromosomes are of two types: macrochromosomes and micro chromosomes. However, there is no strict boundary between the two types and the distinction is entirely arbitrary (Hammer, 1970; Takagi and Sasaki, 1974; Biederman et al., 1980; Stock and Bunch, 1982). The sex chromosomes in birds are designated Z and W, unlike the mammalian X and Y. Also, unlike mammals, the females are heterogametic, possessing one Z and one W chromosome, while the males are homogametic containing two Z chromosomes. It has been shown that related bird species have very similar karyotypes (Hammer, 1970; Takagi et al.,

1972; De Lucca, 1978; De Boer and Van Bocxstaele, 1981; Ryttman and Tegelstrom, 1981; Stock and Bunch, 1982).

Numerous methods for obtaining and staining chromosomes for karyotyping are known (Appendix I). Nonbanded chromosomes are not pretreated and the stain is applied directly to the chromosome spreads resulting in preparations that appear evenly stained and without bands. Banded chromosomes have the chromosome spreads pretreated before applying the stain. These chromosomes are not evenly stained, but show transverse dark and light bands along their length.

Chromosomes have been obtained from many different tissues with varying degrees of success. Blood cultures were used by Takagi et al. (1972), Takagi and Sasaki (1974), Au et al. (1975), De Boer (1976), Biederman et al. (1980), De Boer and Belterman (1981) and De Boer and Van Bocxstaele (1981) as a source of cells for their chromosome work. The complete karyotypes presented by these researchers were stained with the conventional nonbanded chromosome techniques. In addition, Takagi and Sasaki (1974) and De Boer and Belterman (1981) gave partial karyotypes which were banded. Belterman and De Boer (1984) presented the karyotypes of 55 species of birds using lymphocytes to obtain the chromosomes. They note that their results are generally poor because "the techniques for culturing the lymphocytes of birds have not

reached the stage of sophistication they have in mammals." The karyotypes presented are nonbanded. Belterman and De Boer (1984) believe that even nonbanded karyotypes contribute to the knowledge of the basic karyology of the various orders and families "because the data so far available for most of the larger avian taxa are extremely poor."

Some researchers listed in Appendix I used other body tissues to obtain chromosomes. Bloom et al. (1972) used the allantoic sac treated in situ with colcemid and obtained the chromosomes by squashing this tissue. The chromosomes shown were not banded and no attempt was made to karyotype the chick embryo. De Lucca (1978) injected animals with colchicine solution, euthanatized them and treated small pieces of spleen, liver and gonads by keeping them in distilled water for 10 minutes before fixing them in 50% acetic acid. Squash preparations were then made. The resultant chromosomes were poor and demonstrated what Belterman and De Boer (1984) calls the colcemid effect, ie., the chromatids were separated. Hammer (1966; 1970) and Hammer and Herlin (1975) also used a squash technique to obtain chromosomes. Tissues were pretreated with a hypotonic solution, then with colchicine, fixed in acetic acid and alcohol and squashed. The metaphase spreads produced were such that it was difficult to determine the exact chromosome numbers.

In the class Aves there are over 9100 species (Clements, 1978), of which less than 2% have been karyotyped (Takagi and Sasaki, 1974), and of these, fewer again have been karyotyped using improved culturing, harvesting and staining techniques. Although the introduction of chromosome banding techniques has led to many mammalian cytogenetic studies, few similar studies have been performed on bird cells (Carlenius *et al*., 1981), except for the galliforms.

The Charadriiformes contains three suborders: shorebirds (Charadrii), gulls and terns (Lari) and auks (Alcae). In the suborder Charadrii, two species from the monogeneric family Haematopodidae have been karyotyped. Baker *et al*. (1981), karyotyped a female Variable Oystercatcher (*Haematopus unicolor* Forster) and compared its karyotype to the European Oystercatcher (*Haematopus ostralegus* (L.)), which has been analyzed by Hammer (1970). Cytogenetic studies were also carried out with other birds within this suborder by Hammer (1970). The species included: the Lapwing (*Vanellus vanellus* (L.)), the Ringed Plover (*Charadrius hiaticula* (L.)), the Snipe (*Gallinago gallinago* (L.)), the Curlew (*Numenius arquata* (L.)), the Redshank (*Tringa totanus* (L.)) and the Avocet (*Recurvirostra avosetta* L.). Ryttman *et al*. (1979) presented partial karyotypes of four species belonging to the suborder Lari: the Herring Gull (*Larus argentatus*

Pontopiddan), the Lesser Black-backed Gull (*Larus fuscus* (L.)), the Great Black-backed Gull (*Larus marinus* L.) and the Common Gull (*Larus canus* (L.)). Ray-Chaudhuri (1973) published data in the form of idiograms on relative arm lengths of the first fifteen chromosomes of six larid species, namely: the Common Gull, Herring Gull, Black-headed Gull (*Larus ridibundus* L.) and three species of terns: Arctic Tern (*Sterna paradisaea* Pontopiddan), Common Tern (*Sterna hirundo* L.) and Least Tern (*Sterna albifrons* Pallas). The Herring Gull was also karyotyped by Itoh *et al*. (1969). Belterman and De Boer (1984) gave a summary of the bird species that had chromosome preparations made from gonadal tissue. However, to date, no one has cultured tissue or karyotyped somatic cells from members of the suborder Alcae. The present study was therefore undertaken to develop a reliable method for obtaining mitotic chromosomes for karyotyping, to analyse the chromosomes of the Common Murre (*Uria aalge* Pontopiddan), the Razorbill (*Alca torda* L.) and the Atlantic Puffin (*Fratercula arctica* (L.)), and to compare them with the Herring Gull, Great Black-backed Gull and Black-legged Kittiwake (*Rissa tridactyla* L.). The data thus obtained might then be used to provide an insight into the relationships between these two suborders.

MATERIALS AND METHODS

Collections:

Eggs (two L. argentatus, two L. marinus, one R. tridactyla, two U. aalge, one A. torda and two F. arctica) were collected from Gull Island in the Witless Bay Seabird Sanctuary (47°16'N; 52°46'W), approximately 33km south of St. John's, Newfoundland during the mid-incubation period of the breeding season, 1980. The eggs were returned to the laboratory and disinfected by immersion in 95% ethanol for 5 to 10 minutes.

Primary Explant Procedures:

The following procedures were carried out in a laminar flow cabinet under sterile conditions. Following immersion in 95% ethanol, the eggs were wiped dry with gauze, the shells were cracked in the air space region and the embryos were removed with forceps. Supported by a second pair of forceps,the embryos were tipped into glass petri dishes. The yolk sacs were separated from the embryos which were then placed into a second glass petri dish containing 5ml of Roswell Park Memorial Institute Medium 1640, supplemented with 20% fetal calf serum (RPMI-FCS) (Appendix II). Using two disposable scalpels manipulated in a criss-cross manner, the embryos were cut up and then added, using a pasteur pipette, to $25cm^2$ Corning tissue culture flasks. Three ml of RPMI-FCS were added to each

flask. This procedure is outlined in Fig.1. The number of flasks set up from each embryo is listed in Table 1. Since the embryos of L. argentatus and L. marinus, were at an advanced stage of development and had feathers, only pectoralis muscle tissue was used to establish the explant. Prior to dissection, the feathers were removed using forceps to avoid the inclusion of feather particles in the tissue culture flasks. All cultures were incubated at 37°C in a humidified, 8% carbon dioxide atmosphere in a NAPCO water-jacketed incubator. After allowing the cultures to incubate for 48 hours to permit the cells to settle and attach to the plastic surface, the medium was poured off and discarded and 5ml of fresh RPMI-FCS was added to each flask. The attached cells were observed every 24 hours using a Nikon inverted phase microscope with a 10X objective and 10X oculars.

Subculturing using Trypsin-Ethylenediaminetetraacetic acid (EDTA):

The subculturing procedure is outlined in Fig.2. When confluent monolayers of fibroblast-like cells had formed, the medium was poured off and discarded. The flasks were then rinsed three times with 5ml of Hanks balanced salt solution, calcium and magnesium free (BSS), to remove any fetal calf serum. Residual fetal calf serum would have inhibited trypsin activity. A fourth aliquot of Hanks BSS

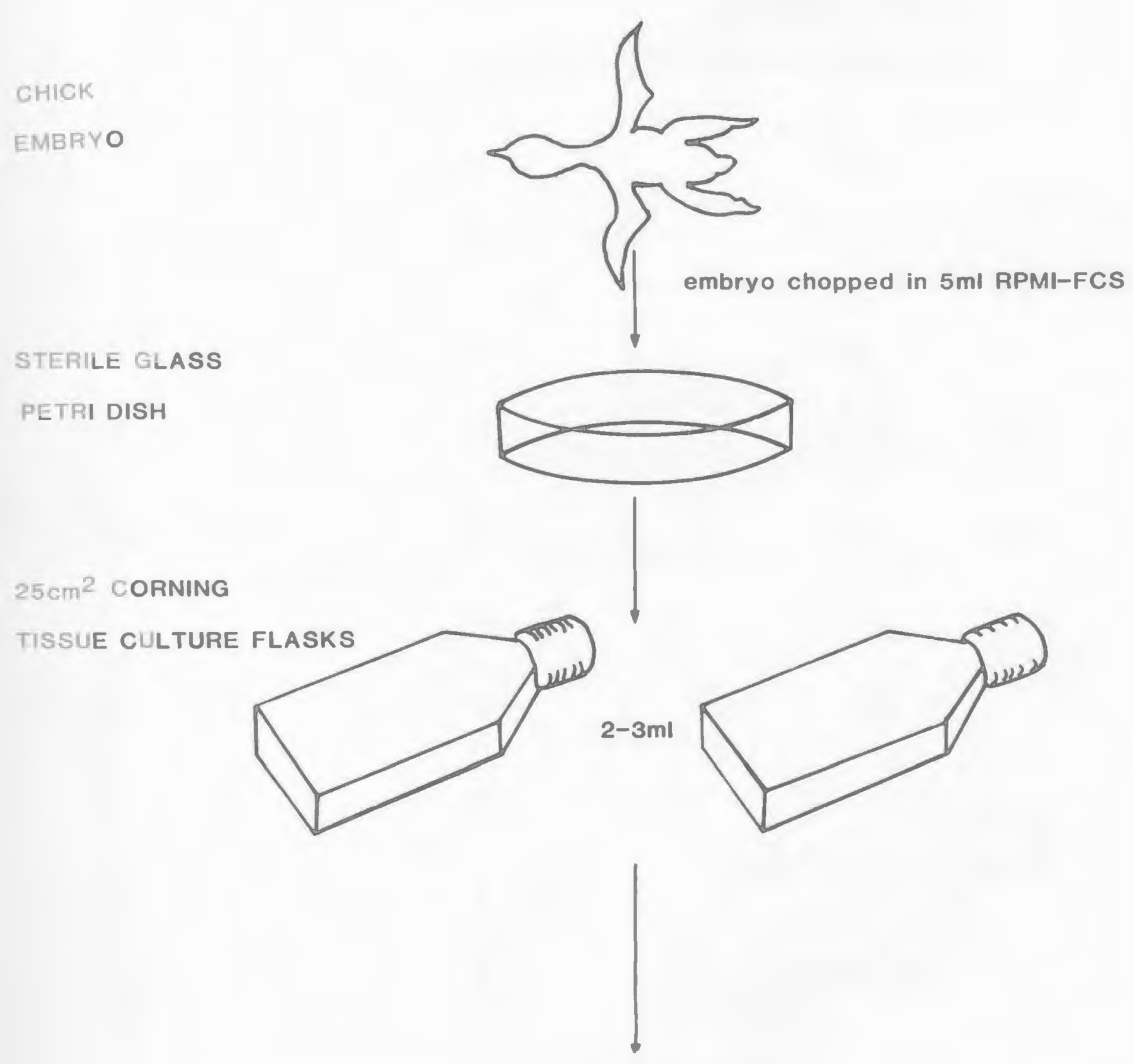

FIG. 1 PRIMARY EXPLANT PROCEDURE

Table 1. The total number of flasks set up from each embryo and the number of flasks containing attached cells after a 48 hour period.

SPECIES	NUMBER OF FLASKS	NUMBER WITH ATTACHED CELLS
L. argentatus #1	16	14
L. argentatus #2	12	12
L. marinus #1	12	12
L. marinus #2	3	1
R. tridactyla	4	4
U. aalge #1	2	1
U. aalge #2	4	4
A. torda	3	3
F. arctica #1	2	2
F. arctica #2	3	2

* Number of flasks set up is a reflection of the time available and also subsequent subculturing.

CONFLUENT

MONOLAYER

usually 48 hr. post set up

medium discarded
monolayer washed with Hanks BSS
last wash remained on cells 5min.

1ml 1X trypsin-EDTA solution added
flask incubated at 37 C, 3-5 min.

1ml cells   trypsin-EDTA drawn from flask

0.5ml added
to each flask

TWO 25 cm^2 CORNING
TISSUE CULTURE
FLASKS

5ml RPMI-FCS added to each flask

flasks incubated at 37 C humidifed, 8% CO2 atmosphere

FIG. 2 SUBCULTURING THE PRIMARY CULTURE

was allowed to remain in the flasks for 3-5 minutes before being discarded. One ml of 1X trypsin-EDTA solution was added to each flask followed by incubation at 37°C for 3-5 minutes to enable the cells to detach from the flask's surface. Any cells that remained attached were released by tapping the side of the flask. The trypsin served to detach cells from the flask wall, while the EDTA prevented cell aggregation by rendering soluble the magnesium and calcium in the cell-cementing material. From the cell suspensions produced, 0.5ml was removed and added to another 25cm^2 tissue culture flask. Five ml of RPMI-FCS were added to each of the duplicate cultures thus produced. The flasks were then incubated at 37°C.

Subculturing without Trypsin-EDTA:

A number of monolayers were split without using trypsin-EDTA. The cells and sometimes small tissue fragments were removed by vigorously shaking the flask and pouring the contents into a second 25cm^2 Corning tissue culture flask. Not all the cells, however, were detached from the original flask. The cells remaining were replenished with 5ml of RPMI-FCS medium. Both flasks were then incubated at 37°C.

Chromosome Preparation:

The subcultures were microscopically examined daily using an inverted phase microscope for cells in the metaphase stage of mitosis. When 50 or more such cells were counted in the entire flask, a chromosome harvest was carried out. The procedure is detailed in Fig. 3. Fifty or 75ul of colcemid (to make a final colcemid concentration of 0.05 or 0.075ng/ml) were added to the RPMI-FCS medium to arrest the cells in metaphase. The flask was incubated at 37°C for 15-20 minutes. Colcemid and medium were then poured off into a 15ml conical centrifuge tube after which the flask was rinsed two to three times with two to three ml of Hanks BSS. The last rinse was allowed to stand for 5 minutes in the flask before decanting. Two ml of 1X trypsin-EDTA solution was added to the flask and the flask was re-examined for detaching cells. The first cells to come off following the addition of the trypsin-EDTA solution were poured into a second conical centrifuge tube and the flask rinsed with Hanks BSS which was also poured into that tube. Both tubes were centrifuged at 200Xg for 8 minutes. Since not all the cells were removed from the flask for the chromosome harvest, 5ml of RPMI-FCS was added to the flask which was returned to the incubator for continued cell growth. Supernatants from the tubes were removed with a pasteur pipette and the pellets resuspended in 1ml of hypotonic

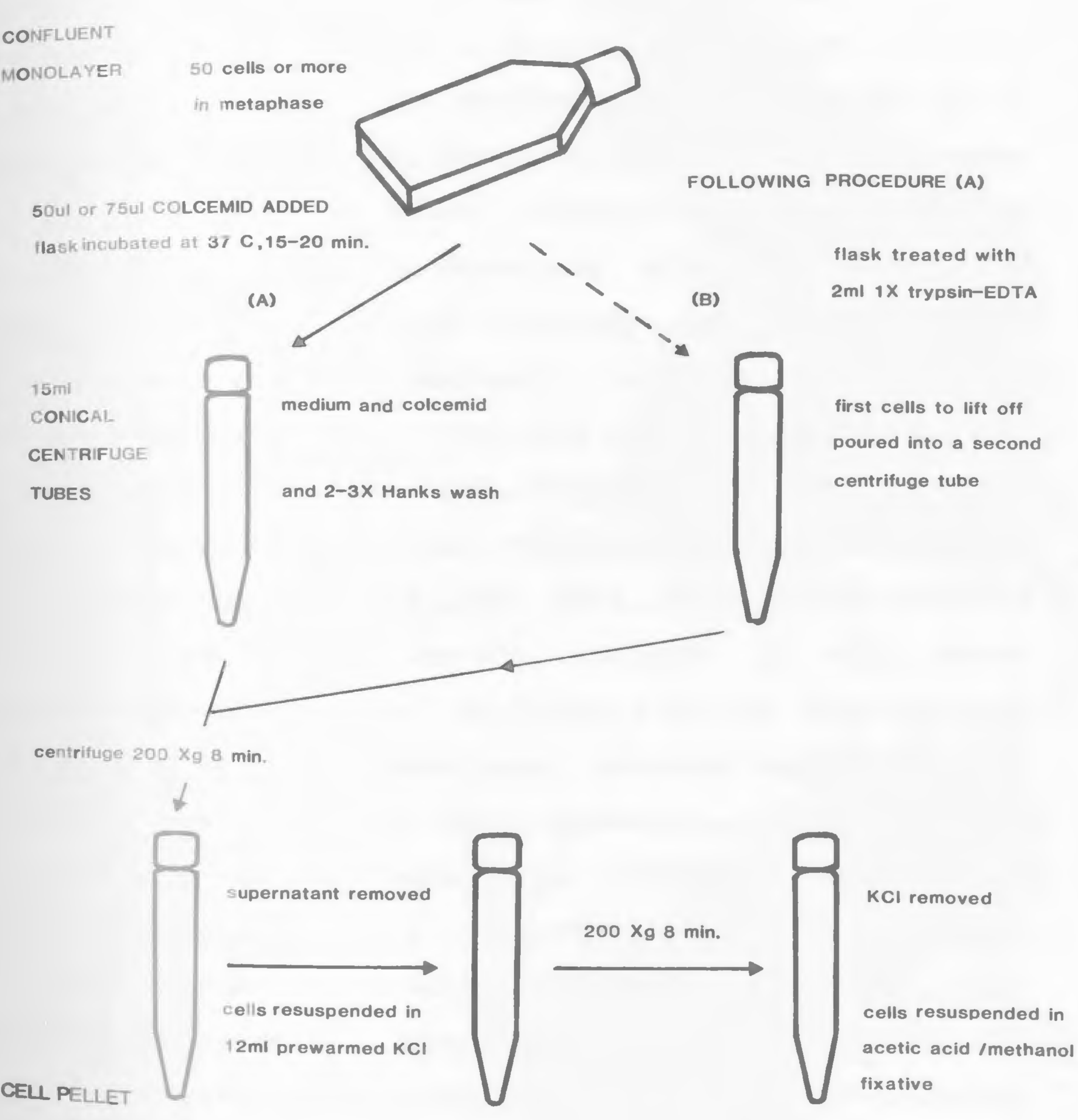

FIG. 3 CHROMOSOMAL HARVEST

solution using either 75mM KCl or 1.1% sodium citrate at 21°C or prewarmed to 37°C. The hypotomic solution was slowly added to the tube and the tube was then left at 21°C or placed in a 37°C waterbath for 15-20 minutes. Following treatment in the hypotonic reagent, the cells were centrifuged at 200Xg for 8 minutes. Following removal of the supernatant, fixative (acetic acid and absolute methanol, 1:3) was slowly added and the cell suspension gently agitated to avoid clumping the nuclei. Since evaporation of the methanol altered the acetic acid/methanol ratio, the fixative was freshly prepared before use. To eliminate cell debris, the fixative was changed 4 to 5 times. The first aliquot of fixative remained in contact with the cell suspensions for 30 minutes at room temperature or overnight at 4°C, after which time centrifugation at 200Xg for 8 minutes was carried out. The supernatant was decanted and the cell pellets resuspended in 15ml fixative. This washing procedure was repeated twice more. After the last change of acetic acid/methanol fixative, the cells were resuspended in 0.5 to 1.0ml of fixative. From the cell material thus prepared, two preparations were made: (1) slide preparations (one drop of the chromosome suspension added to a slide containing one drop of 10% acetic acid) for Giemsa banding (G-banding) and, (2) coverslip preparations (one drop of the chromosome suspension added to a coverslip containing one drop of 10% acetic acid) for

fluorescent Q-banding. Giemsa banding refers to pretreating chromosomes with trypsin, which is believed to hydrolyse certain portions of the chromosome and expose the DNA (Wang and Federoff, 1972). This DNA is then stained with a dye such as Giemsa, Wright's or Leishman's stain. With this staining procedure, each chromosome has a characteristic banding pattern, thus making chromosome pairing easier. With Giemsa staining the pretreatment with trypsin is omitted and the chromsomes are evenly stained without bands. Giemsa banding was chosen rather than Giemsa staining to help pair homologous chromosomes by their banding patterns, and also to see if the banding patterns of the first 13 pairs of autosomes and the sex chromosomes varied among species within the same suborder. Q-banding, involves treating chromosomes with a fluorochrome that was initially thought of having alkylating groups which act on the guanine moieties of the chromosome, thus producing bright fluorescent bands. Q-banding was used to help choose homologous chromosome pairs, to look for different banding patterns among species from the same suborder and to check for chromosomes exhibiting bright fluorescence.

Slide and Coverslip Preparations:

Slides and coverslips were soaked in a solution of 10% acetic acid and 90% ethanol, then wiped dry and streak free with cotton gauze. These items must be scrupulously

clean since dust particles present on the slide or coverslip after it has been stained resemble microchromosomes. For Giemsa banding, one drop of the chromosome preparation was added to one drop of 10% aqueous acetic acid solution on a slide. The drop was blown upon and the slide or coverslip was then placed on a slide warmer at 50°C to dry. Both acetic acid and blowing help to spread the chromosomes. Slides for fluorescent banding were prepared in a similar manner except that the chromosome suspension was placed on a 22mm X 40mm No.1 coverslip which was cellotaped to a slide for support during the staining process. At least two slides were made for fluorescent banding and two for Giemsa banding from each chromosome harvest performed. These slides were examined under a Leitz Ortholux phase contrast microscope, with a 10X phase objective and 10X oculars. Each harvest was then graded as being either (1) a good harvest = the chromosomes were not overlapping and were spread out enough for karyotyping, (2) a poor harvest = the chromosomes overlapped and were surrounded by cytoplasm or (3) a harvest failure = no chromosomes were observed in the preparation.

Giemsa Banding (G-bands):

The method for G-banding was a modification of Seabright (1972) which involved heating the slides for

20-30 minutes at 95°C in a hot air oven. After cooling to room temperature, the slide was immersed in Difco trypsin solution diluted with normal saline, rinsed in normal saline and stained with Wright's stain. The chromosome smears were then examined for banded chromosomes using a Leitz Ortholux photomicroscope, equipped with a 100X oil immersion objective and 10X ocular magnification.

Fluorescent Banding, Photography and Karyotyping:

The method for Q-banding followed was that of Miller et al., (1972). The slide-coverslips were immersed in 0.01% aqueous Atebrin stain for about 20 minutes, followed by rinsing for one to two minutes under running tap water. The coverslip was then carefully removed from the slide and the cellotape removed from the coverslip. A few drops of tris-maleic buffer pH 5.6 were added to a clean slide which had been previously soaked in a solution of 10% acetic acid and 90% ethanol. The coverslip carrying the chromosome spreads was inverted onto the buffer. Excess buffer was removed by blotting with gauze, following which the coverslip was sealed to the slide with nail polish. Fluorescent photomicroscopy was carried out on a Leitz Ortholux photomicroscope under epi-illumination, with an HBO 200-W mercury vapor lamp, BG12 exciter filter, and 490nm barrier filter. A 100X nPl oil immersion objective and 10X oculars were used. Photographs were taken on Kodak

Panatomic X 35mm film, developed in Kodak Microdol X diluted 1:3 with tap water at 21°C for 13 minutes. Enlargements were made on Kodabrome F 5 paper, developed in Kodak SII activator and Kodak Ektamatic S30 stabilizer, rinsed in running tap water and fixed in 1% Kodak rapid fixative for 10 minutes. Following enlargement, the final magnification of each photograph was approximately 2000X.

Partial karyotypes were made for each bird species and idiograms were constructed from the karyotypes. Since the distinction between macrochromosomes and microchromosomes is arbitrary (Stock and Bunch, 1982), in this project the chromosomes which were largest and gave the maximum brightness under fluorescence were karyotyped. In all species examined, this included 13 pairs of autosomes and the pair of sex chromosomes. The chromosomes were arranged from the largest to smallest. The p (short) and q (long) arms and the c (centromere) regions were measured. The total length of the 13 largest haploid chromosomes was determined. The p, q and c areas were expressed as a percentage of the total length. The mean of the percentage for each segment was used to construct the idiogram.

Various terminologies have been used when describing the chromosomes within a karyotype. The majority, however, relate to the centromere position. The terminology used in describing the chromosomes in this study follows that of Levan _et al_., (1964): the term metacentric is given to chromosomes having an arm ratio of 1:1.0-1.7;

submetacentric refers to an arm ratio of 1:1.7-3.0; subtelocentric are ratios above 1:3.0 and acrocentric chromosomes are those in which there are no distinct short arms consistently visible (Fig. 4).

Blood Cultures:

Prior to, and simultaneously with, cell culture work, several unsuccessful attempts were made to obtain chromosomes from peripheral blood lymphocytes. Blood was collected from the brachial vein of two adult Domestic Fowls (*Gallus gallus* L.) and one Herring Gull (*L. argentatus*), using a heparinized vacutainer with a 20 gauge needle. One-quarter ml of blood was added to 5ml of RPMI-FCS and 0.05ml phytohaemagglutin solution. Chromosomes were harvested from these blood samples at 48, 72, and 96 hours. Metaphases were arrested with colcemid treatment at 37°C for 25 minutes.

Other unsuccessful attempts were made at culturing blood lymphocytes by separating them from the other blood cells using Ficoll Paque solution according to the method outlined in the Pharmacia Fine Chemicals booklet supplied with the Ficoll Paque kit. Three or four drops of the layer containing lymphocytes were added to 5ml of culture medium. However, as cell culture work proved to be successful, further attempts to grow lymphocytes were discontinued.

Term	Arm	Idiogram	p/q Arm Ratio
Metacentric	p		1.0
	q		1.0 - 1.7
Submetacentric	p		1.0
	q		1.7 - 3.0
Subtelocentric	p		1.0
	q		3.0 > 3.0
Acrocentric	p		
	q		

FIG. 4 TERMINOLOGY and RATIOS

Note a subtelocentric chromosome has a q arm segment ratio greater than 3.0.
Also an acrocentric chromosome lacks a p arm segment.

Centromere Banding (C-Banding):

The chromosomes are pretreated before staining so that only the centromere of the chromosome will absorb the stain.

Giemsa Banding (G-Banding):

The chromosomes are pretreated with trypsin and stained with Wright's, Giemsa or Leishman's stain.

Giemsa Staining:

The chromosomes are not pretreated before the stain is applied thus the chromosomes appear evenly stained and without bands.

Fluorescent Banding (Q-Banding):

The chromosomes are treated with a fluorchrome that contains alkylating groups.

RPMI-FCS:

Roswell Park Memorial Institute Medium 1640 supplemented with 20% fetal calf serum.

Trypsin-EDTA:

Trypsin with Ethylenediaminetetraacetic acid

RESULTS AND DISCUSSION

Successful primary explants were obtained from all of the 10 Charadiiformes embryos collected. Table 1 details the total number of flasks in which cells or tissue fragments were attached to the flask's surface after 48 hours. Two flasks each of L. argentatus #1 and L. marinus #2 and one of U. aalge #1 were discarded because of what appeared to be bacterial contamination. Though F. arctica #2 showed no contamination, tissue fragments did not settle and later disintegrated and died. As seen in Table 1, the establishment of primary explants was a success, with cells or tissue fragments of all ten embryos attaching to the flasks. In total, 90.2% had the explants growing within 48 hours.

Subculturing:

Cells were subcultured both with and without trypsin (Table 2). Only flasks which formed confluent monolayers were subcultured (Tables 1 and 2). For example, cells of L. argentatus became attached in fourteen flasks within 48 hours. Of these fourteen, seven were subcultured using trypsin and five without using the enzyme. Two flasks had insufficient cells for subculturing. Some flasks were subcultured a number of times depending on the rate of monolayer formation (Table 2). The single successful culture of L. marinus #2, for example, divided and grew rapidly so as to permit subculturing twice with trypsin

Table 2. The numbers of subcultures, harvests and slides made for each avian species.

SPECIES	WITH TRYPSIN	WITHOUT TRYPSIN	HARVESTS	SLIDES
L. argentatus #1	7	5	16	64
L. argentatus #2	8	4	30	120
L. marinus #1	4	4	18	72
L. marinus #2	2	3	7	28
R. tridactyla	2	4	16	64
U. aalge #1	3	4	15	60
U. aalge #2	1	4	8	32
A. torda	2	3	5	20
F. arctica #1	2	3	8	32
F. arctica #2	1	2	9	36

and three times without trypsin. Overall, the subculturing procedure, with or without trypsin, was successful.

Chromosome Preparations:

In order to obtain good chromosome preparations, colcemid concentrations and times, hypotonic solutions, times and temperatures were varied (Appendix IIIA). A minimum of four slides were made and graded microscopically before staining (Table 3). If more than four slide preparations were required after staining, the cell suspensions were spun down, fresh fixative added and smears made. From a total of 132 chromosome harvests there were 32 complete failures (no chromosomes were observed in the preparations), 58 harvests contained chromosomes that overlapped and were surrounded with cytoplasm and 42 harvests contained chromosomes that were sufficiently spread for karyotyping. The various combinations of (1) final colcemid concentration (2) time in colcemid (3) hypotonic solution used (4) time in hypotonic solution and (5) the temperature of the hypotonic treatment, and their results are listed in Table 4. The table reveals that methods H, K, O, P and Q produced chromosomes that were evaluated as being good enough for karyotyping, with method Q apparently being the most reliable procedure. Because the sample size was small, the 19 methods used could bear further investigation to determine their

Table 3. The chromosome preparations for each bird species and the percent success (in parentheses).

SPECIES	TOTAL	+GOOD	*POOR	-FAILURE
L. argentatus #1	16	4 (25.0)	9 (56.3)	3 (18.8)
L. argentatus #2	30	10 (33.3)	16 (53.3)	4 (13.3)
L. marinus #1	18	10 (55.5)	6 (33.4)	2 (11.1)
L. marinus #2	7	1 (14.3)	1 (14.3)	5 (71.4)
R. tridactyla	16	4 (25.0)	10 (62.5)	2 (12.5)
U. aalge #1	15	3 (18.8)	4 (25.0)	8 (56.2)
U. aalge #2	8	3 (37.5)	3 (37.5)	2 (25.0)
A. torda	5	2 (40.0)	1 (20.0)	2 (40.0)
F. arctica #1	8	2 (25.0)	5 (62.5)	1 (12.5)
F. arctica #2	9	3 (33.3)	3 (33.3)	3 (33.3)
TOTAL	132	42	58	32

+ GOOD HARVESTS = Chromosomes not overlapping; sufficient spread for karyotyping

* POOR HARVESTS = Chromosomes overlapping and surrounded by cytoplasm

- HARVEST FAILURE = No chromosomes observed

Table 4. A summary of the harvesting methods, the total numbers and the grading of each harvest.

	HARVEST METHOD	NUMBERS	TOTAL	GOOD	POOR	FAIL
A	0.05 ng/ml colcemid 30 min., KCl 15 min. at 21 C	1-4	4	0	4	0
B	0.05 ng/ml colcemid 10 min., KCl 15 min. at 21 C	5,6	2	0	2	0
C	0.075 ng/ml colcemid 20 min., KCl 15 min. at 21 C	7-9	3	0	3	0
D	0.05 ng/ml colcemid 15 min., KCl 15 min. at 21 C	10-11	3	0	3	0
E	0.05 ng/ml colcemid 10 min., KCl 17 min. at 37 C	12-13	2	0	0	2
F	0.05 ng/ml colcemid 15 min., KCl 15 min. at 37 C	14,15	2	0	2	0
G	0.05 ng/ml colcemid 5 min., KCl 17 min. at 37 C	16	1	0	0	1
H	0.075 ng/ml colcemid 15 min., KCl 17 min. at 37 C	17,26-39	15	3	9	3
I	0.075 ng/ml colcemid 20 min., KCl 18 min. at 21 C	18,19	2	0	2	0
J	0.05 ng/ml colcemid 5 min., KCl 20 min. at 21 C	20-23 46-51	10	0	10	0
K	0.075 ng/ml colcemid 15 min., KCl 18 min. at 37 C	24,25	2	2	0	0

Table 4 continued

	HARVEST METHOD	NUMBERS	TOTAL	GOOD	POOR	FAIL
L	0.075 ng/ml colcemid 30 min., KCl 18 min. at 21 C	40,41	2	0	2	0
M	0.05 ng/ml colcemid 15 min., KCl 20 min. at 21 C	42-44	3	0	3	0
N	0.075 ng/ml colcemid 5 min., KCl 20 min. at 37 C	45	1	0	0	1
O	0.075 ng/ml colcemid 15 min., KCl 20 min. at 21 C	52-54	3	1	1	1
P	0.075 ng/ml colcemid 5 min., KCl 20 min. at 21 C	55-61	12	1	8	3
Q	0.075 ng/ml colcemid 20 min., NaCit 18 min. at 21 C	62,63 66-77 81-87 89-103 104-132	72	36	20	16
R	0.075 ng/ml colcemid 20 min., NaCit 15 min. at 21 C	64,65	2	0	1	1
S	0.075 ng/ml colcemid 15 min., NaCit 18 min. at 21 C	78-80 88	5	0	0	5
	TOTAL		132			

reproducibility.

In addition to an examination of the various harvesting techniques, one must also consider the length of time these cells were growing in tissue culture, because normal cells can be grown for only short periods of time in flasks. Table 5 lists the number of days in culture, the number of harvests performed on a species on a particular day and the number of good harvests obtained. The data from this table show that good chromosome harvests of larid cells may be obtained in eighteen to fifty-seven days of culture and for alcid cells in from fourteen to fiıty-three days.

Seventy-two chromosome harvests were done using method Q. Referring to Table 5 and Appendix IIIB, success of a harvest using method Q appears to be independent of time in culture. As the table and appendix indicate, good harvests were obtained from cells that grew from twenty to fifty-seven days in culture. It may be possible to obtain a good harvest earlier than twenty days using method Q.

It should be noted that subsequent work with human cancer cells has used 100%, rather than 10%, acetic acid to aid chromosome spreading. This alteration in technique has achieved considerable success. Extrapolating such results to cells other than human is not always possible. It does, though, suggest an area for further investigation.

Table 5. Days in culture yielding good chromosome harvests for each bird species.

SPECIES	DAYS IN CULTURE (GOOD HARVEST/TOTAL HARVESTS THAT DAY
L. argentatus #1	18(2/2) 31(2/2)
L. argentatus #2	18(3/14) 31(1/2) 39(2/2) 43(2/2) 46(2/2)
L. marinus #1	39(2/2) 43(2/2) 47(2/2) 54(2/2) 57(2/2)
L. marinus #2	52(1/1)
R. tridactyla	20(1/2) 25(1/1) 26(2/2)
U. aalge #1	42(2/2) 52(1/1)
U. aalge #2	25(1/1) 39(1/1) 48(1/1)
A. torda	21(2/2)
F. arctica #1	14(1/1) 24(1/1)
F. arctica #2	22(2/3) 53(1/1)

Staining:

Of the two banding techniques attempted, only the fluorescent or Q-banding was successful although some metaphases appeared "fuzzy" and slightly out of focus, a problem which might have been eliminated by changing the fixative more often. This information however, was not known when the avian chromosomes were harvested. At times, the fixative must be changed as many as ten times to eliminate the fuzziness.

Photography and Karyotyping:

After carefully examining each slide for banded, intact chromosome spreads, the photomicrographs were prepared. Prints were not prepared of the chromosome spreads from all the embryos. Spreads from *L. marinus* #2, *U. aalge* #2 and *F. arctica* #2 were not used because their chromosomes appeared too fuzzy or too close together for karyotyping. A representative partial karyotype from each bird species is presented in Fig. 5-10. As may be seen from the photograph above the karyotypes (Fig. 5-10) it would be difficult to develop a complete karyotype or establish an exact chromosome number for each of the bird species due to the large number of microchromosomes. The latter are small, and hard to distinguish and pair. Biederman *et al*. (1980) suggested that to obtain a

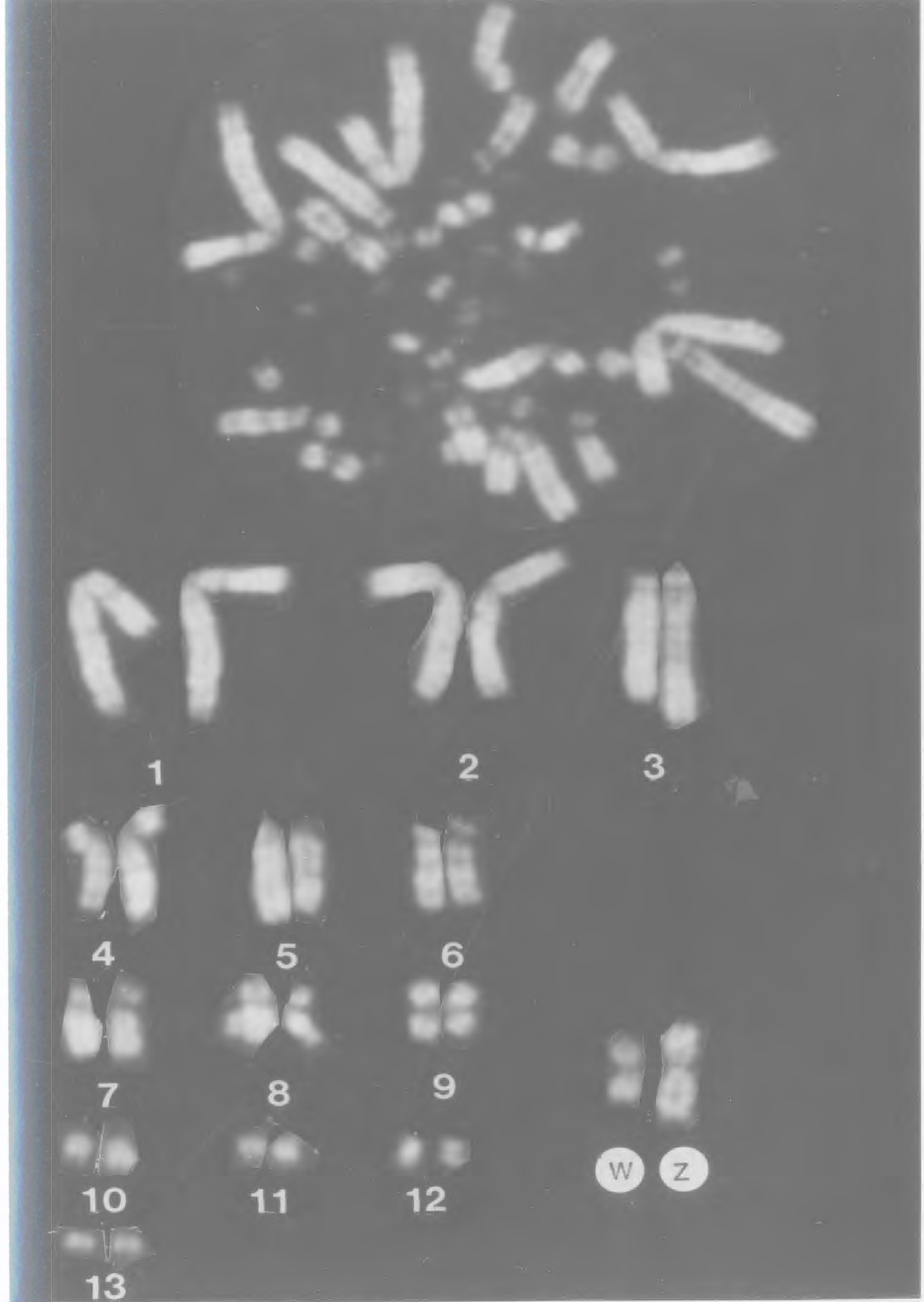

Fig. 5 Q-banded partial karyotype of female Herring Gull (L. argentatus)

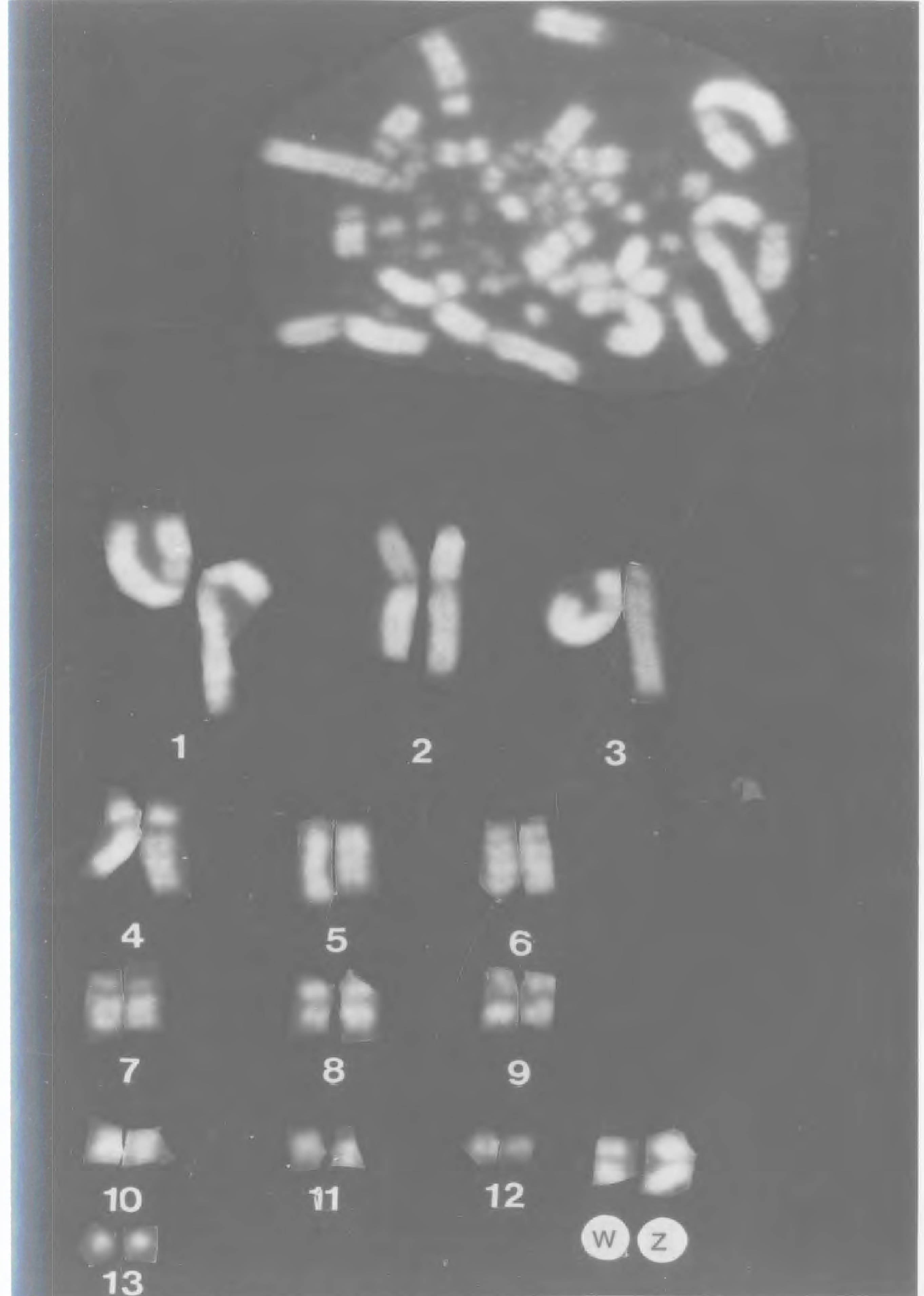

Fig. 6 Q-banded partial karyotype of female Great Black-backed Gull (*L. marinus*)

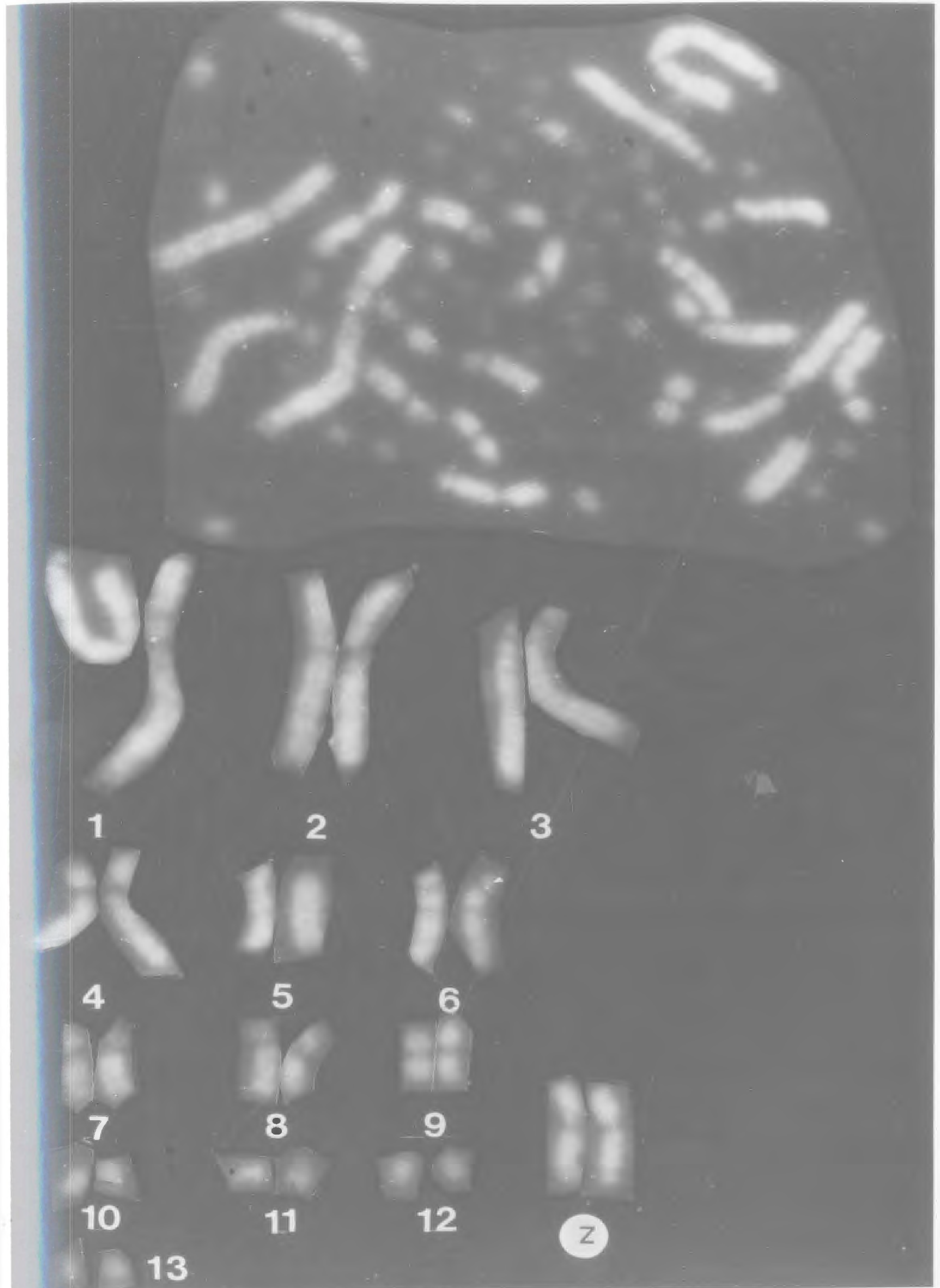

Fig. 7 Q-banded partial karyotype of male Black-legged Kittiwake (R. tridactyla)

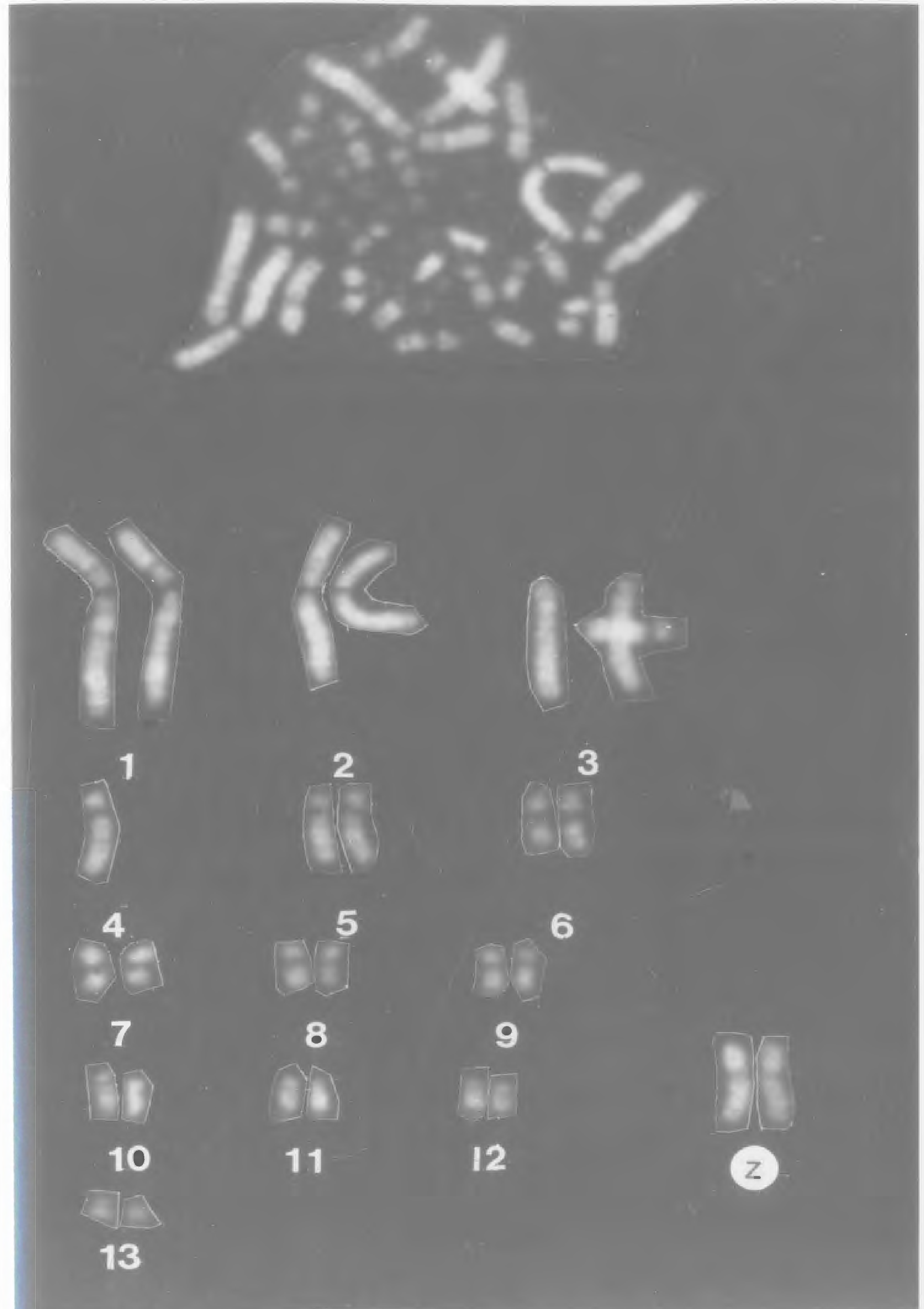

Fig. 8 Q-banded partial karyotype of male Common Murre (U. aalge)

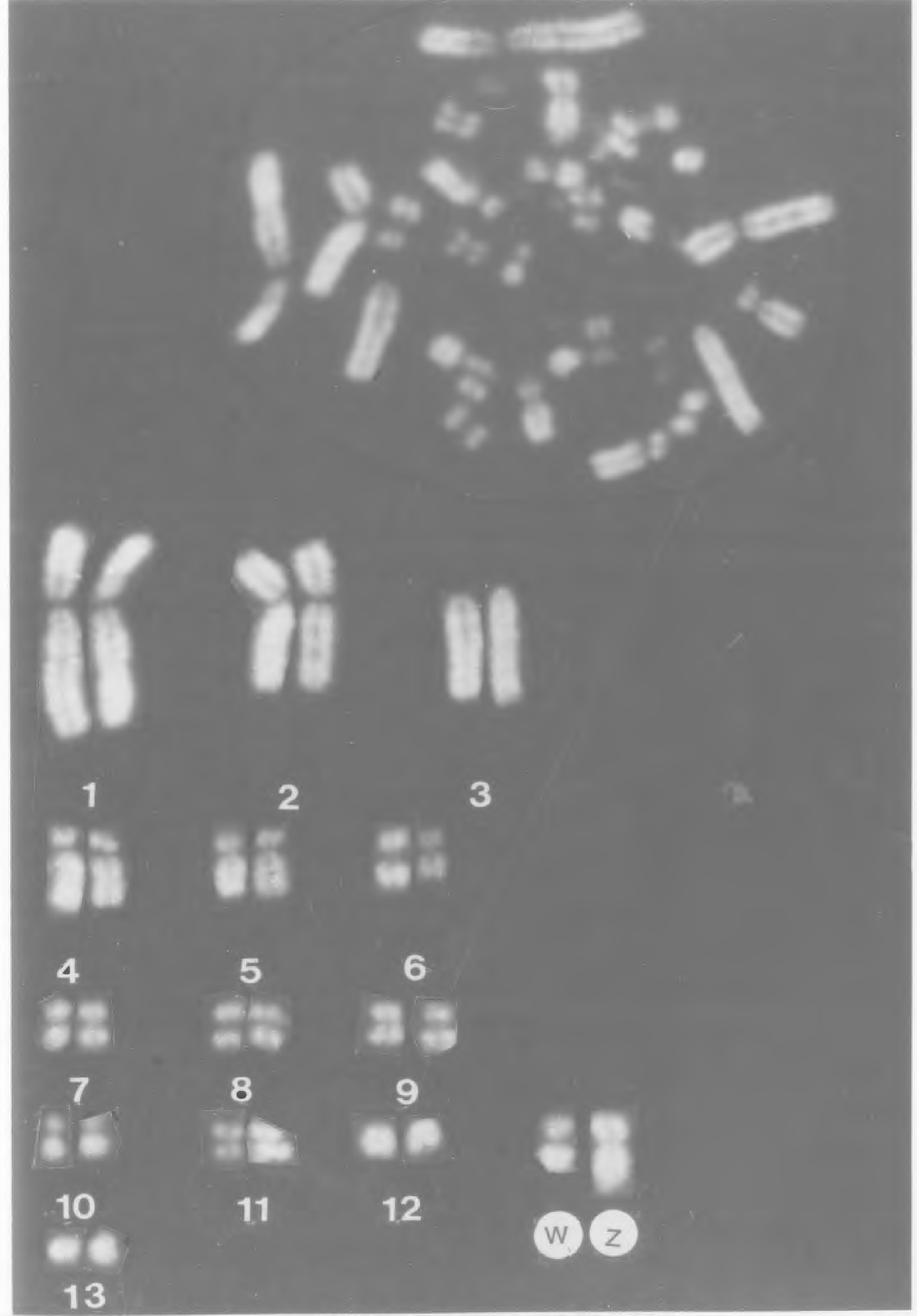

Fig.9 Q-banded partial karyotype of female Razorbill (*A. torda*)

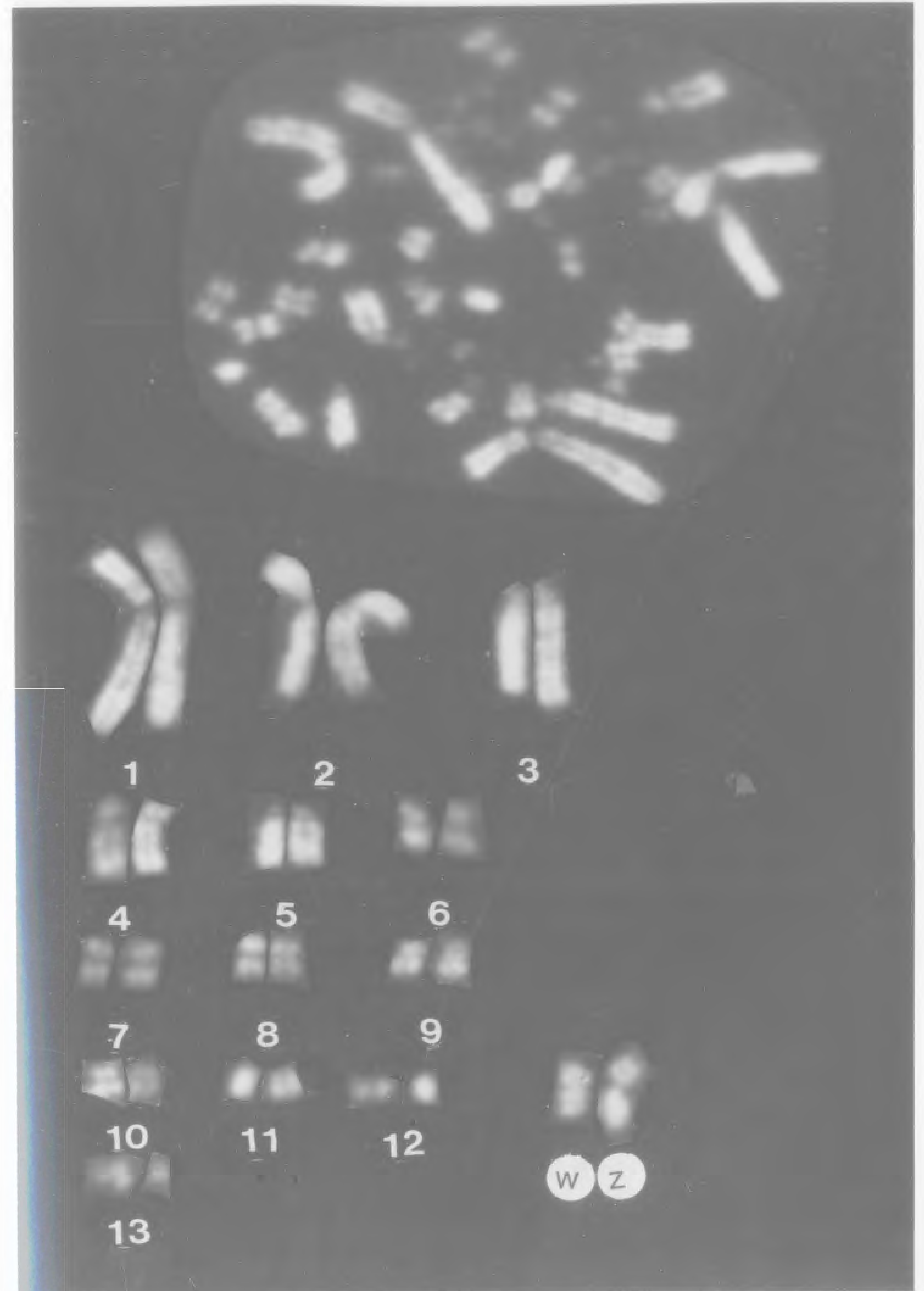

Fig. 10 Q-banded partial karyotype of female Atlantic Puffin (*F. arctica*)

complete karyotype and exact chromosome count of a given species, two or three banding techniques must be used.

The idiograms of the three larids are similar (Fig. 11 a-c), as are their chromosome arm ratios (Appendix IVA). An examination of the ratio of p arm length to q arm length (Table 6) and the Levan terminology of the centromere position between these species (Table 6) reveals great similarities. However minor differences in these three karyotypes are seen in chromosomes 4,7,8 and 10. Chromosome 4 in both L. argentatus and L. marinus is subtelocentric; in R. tridactyla it is submetacentric. The arm ratio for a submetacentric chromosome is 1:1.7-1:3.0. This chromosome in the R. tridactyla has an arm ratio of 1:2.9, just borderline between the two. The subtelocentric chromosome 4 of L. argentatus and L. marinus have ratios of 1:3.5 and 1:3.7 respectively. Chromosome 7 is also submetacentric in R. tridactyla and subtelocentric in the other two species. Chromosome 8 is metacentric in L. marinus and submetacentric in L. argentatus and R. tridactyla. The ratios in these cases are quite distinct. L. marinus has an arm ratio of 1:1.0 while L. argentatus and R. tridactyla have a 1:2.5 and 1:2.3 ratio respectively. Chromosome 10 is metacentric in L. marinus and acrocentric in L. argentatus and R. tridactyla. However, because the chromosomes themselves appear fuzzy and slightly out of focus it is difficult to be precise. The idiograms of the three gulls show L. marinus to have

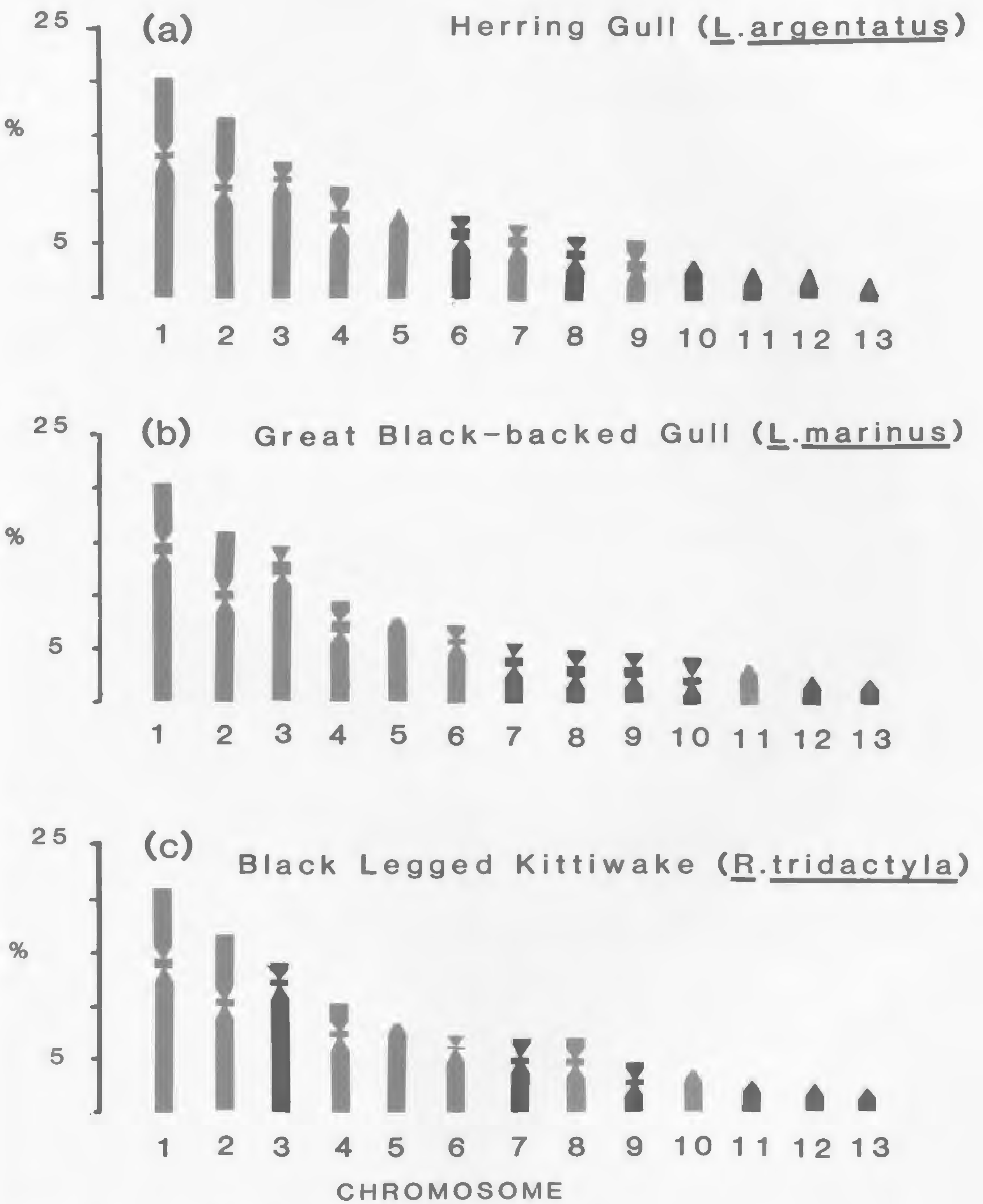

Fig. 11. The idiograms of three larids.

Table 6. The ratio of p arm length to q arm length and the nomenclature for the centromeric position on 13 chromosomes for six seabird species. m = metacentric; sm = submetacentric; st = subtelocentric and a = acrocentric.

Chromosome	L. argentatus	L. marinus	R. tridactyla	U. aalge	A. torda	F. arctica
1	1:1.8 sm	1:2.4 sm	1:1.9 sm	1:2.1 sm	1.1.8 sm	1:1.7 sm
2	1:1.6 m	1:1.5 m	1:1.6 m	1:1.5 m	1:1.7 sm	1:1.8 sm
3	1:9.6 st	1:13.3 st	1:13.0 st	1:8.9 st	– * a	1:10.5 st
4	1:3.5 st	1:3.7 st	1:2.9 sm	1:3.1 st	1:3.1 st	1:4.8 st
5	– a	– a	– a	1:3.4 st	1:2.6 sm	1:6.2 st
6	1:5.1 st	1:4.6 st	1:5.1 st	1:1.4 m	1:1.4 m	1:1.7 sm
7	1:4.0 st	1:3.1 st	1:2.3 sm	1:1.0 m	1:1.1 m	1:1.3 m
8	1:2.5 sm	1:1.0 m	1:2.3 sm	1:1.3 m	1:1.1 m	1:1.2 m
9	1:1.2 m	1:1.4 m	1:1.3 m	1:2.1 sm	1:1.1 m	1:1.4 m
10	– a	1:1.0 m	– a	1:1.8 sm	1:1.1 m	1:1.1 m
11	– a	– a	– a	1:2.7 sm	1:1.4 m	– a
12	– a	– a	– a	– a	– a	– a
13	– a	– a	– a	– a	– a	– a

* = no consistently visible p arm present.

one outstanding difference compared to the L. argentatus or R. tridactyla, namely chromosome 10. This chromosome is metacentric in L. marinus and acrocentric in L. argentatus and R. tridactyla. L. argentatus and R. tridactyla have similar idiograms, with three differences. These are (1) the chromosome 6 in R. tridactyla contains a minute centromere compared to the large centromere of chromosome 6 in L. argentatus cells, (2) chromosome 8 is longer in R. tridactyla because its p arm is longer than the p arm of chromosome 8 in L. argentatus and (3) chromosome 9 is shorter in R. tridactyla than it is in L. argentatus.

In the alcids, chromosome 2 is metacentric in U. aalge and submetacentric in A. torda and F. arctica (Table 6). A second difference is in chromosome 3, which is acrocentric in A. torda and subtelocentric in U. aalge and F. arctica. Thirdly, chromosome 5, which is submetacentric in A. torda and subtelocentric in U. aalge and F. arctica. However chromosome 5 in A. torda and U. aalge from the karyotypes (Fig. 8 and 9) look similar, and chromosome 5 in F. arctica (Fig. 10) appears different because of a short p arm. This is also observed in the arm ratios (Table 6). The ratios are U. aalge 1:3.4, A. torda 1:2.6 and F. arctica 1:6.2. Subtelocentric chromosomes have ratios above 1:3.0; submetacentric chromosomes have ratios of 1:1.7-1:3.0. The subtelocentric chromosome 5 of F. arctica is almost to the point of being acrocentric because of its 1:6.2 ratio. Chromosome 6 is submetacentric

in F. arctica and metacentric in the two remaining species. The next two differences are observed in chromosomes 9 and 10. Both these are submetacentric in U. aalge and metacentric in F. arctica and A. torda. A major difference is observed in chromosome 11 which is acrocentric in F. arctica, submetacentric in U. aalge and metacentric in A. torda. There are similar characteristics in chromosome 11 between A. torda and U. aalge, but chromosome 11 in F. arctica is different. The remaining chromosomes are acrocentric in each species. From the idiograms (Fig. 12a-c) the three species of alcids may be identified by (1) F. arctica has an acrocentric chromosome 11 which is lacking in the other two species and (2) A. torda has an acrocentric chromosome 3 which U. aalge and F. arctica does not possess.

The major differences among the karyotypes (chromosomes) of the larids and the alcids are that the larids have acrocentric chromosomes 5, 11, 12 and 13, whereas, except for F. arctica (which also has an acrocentric chromosome 11) only chromosomes 12 and 13 are acrocentric in the alcids. A further difference is seen in chromosome 3 in that those of the larids have a large centromere, while the centromere is shorter in the alcid chromosome 3. Chromosome 3 in A. torda also lacks a p arm.

The sex chromosomes of these birds are not included in the total chromosome length for determining the ratios, since there would be variation in the ratios between the

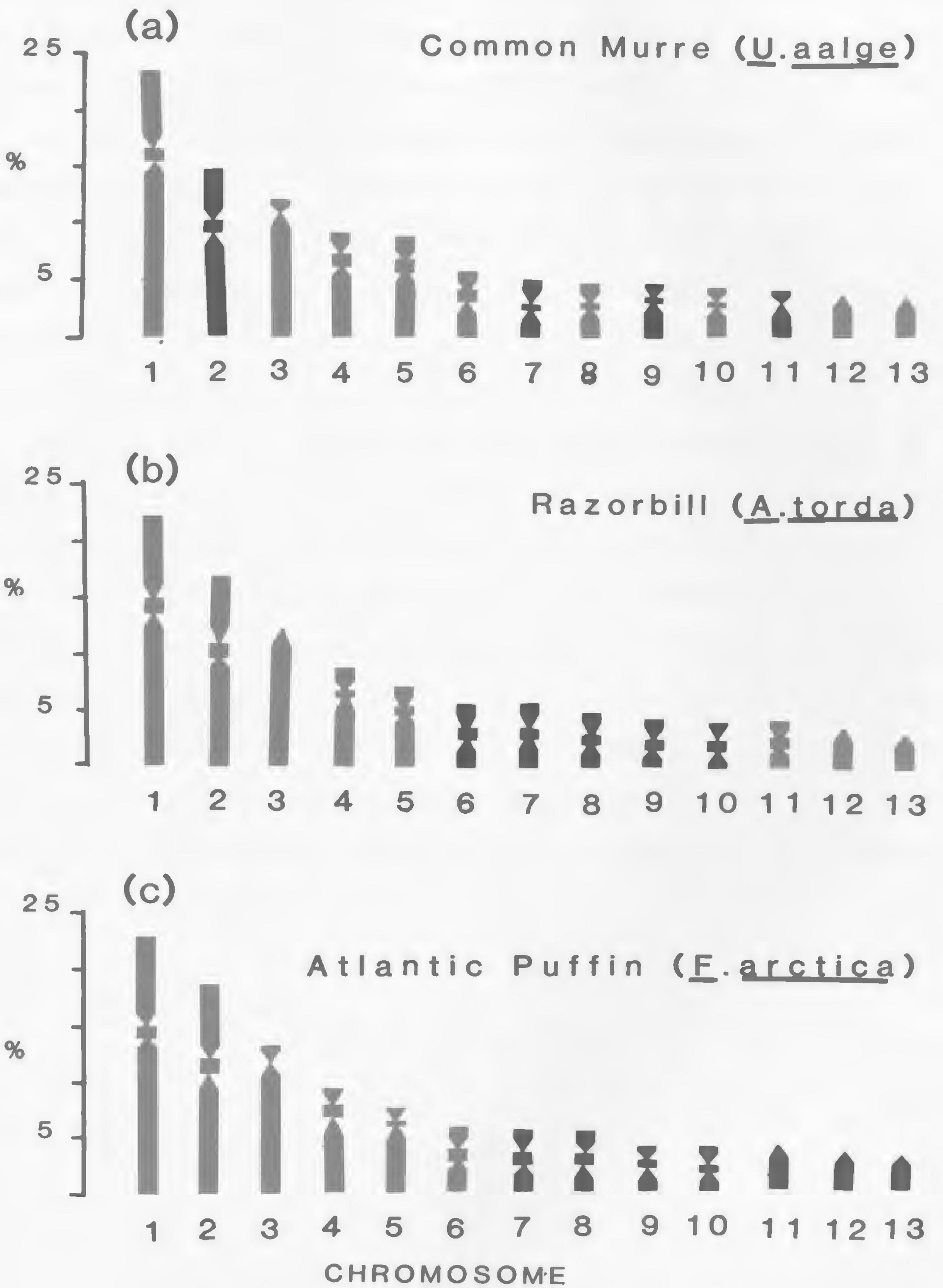

Fig. 12. The idiograms of three alcids.

sexes. Hammer (1970) included one Z chromosome in the haploid genome and Biederman _et al_. (1980) omitted the sex chromosomes in the total chromosome length. However, the sex chromosomes should be included in the discussion. Bird sex chromosomes are designated Z and W instead of X and Y as in mammals. Birds also differ from mammals in that the male is homogametic (having two Z chromosomes) and female is heterogametic (having one Z and one W chromosome).

Of the three larids karyotyped (Fig. 5-7) _L. argentatus_ and _L. marinus_ were both female and _R. tridactyla_ was male. The W chromosome in both species is about 75% of the length of the Z. In the larids both the Z and the W chromosomes appear to be metacentric.

Of the three alcids karyotyped (Fig. 8-10), _U. aalge_ was male and _A. torda_ and _F. arctica_ were female. Again, the W chromosome is about 75% of the length of the Z. The W chromosome appears to be metacentric and similar to the larid W chromosome. However, the alcid Z chromosome appears to be submetacentric.

A number of difficulties can be identified in attempts to karyotype birds. One of these is the selection of the tissue. Belterman and De Boer (1984) point out that culturing blood lymphocytes generally results in rather poor chromosome preparations and detailed banding studies can rarely be carried out. On the other hand, if the bird is to be kept alive, the use of other body tissues is not practical. Appendix I gives some indication of the variety of approaches in the selection of tissues.

The use of bird embryos, though not possible in all cases, does present a viable alternative. Embryonic cells have been used by a number of researchers (Ryttman _et al_., 1979; Baker _et al_., 1981).

Shields (1983) identifies the elements of successful harvest and slide preparation: "In the final analysis, however, there is no substitute for procedures which routinely result in large numbers of excellent spreads of chromosomes on slides which can then be analyzed in detail." He then goes on to describe the difficulties involved in identifying the microchromosomes because of overlapping with larger chromosomes and the failure of researchers to adequately report the microchromosomes. Generally, then, a successful harvesting and slide preparation method would give chromosomes that are spread as much as possible. The success of a harvest depends on the combination of timing of the harvest and chemical pretreatments and their timing.

In order to resolve some of the disputes regarding the classification of chromosomes, attention needs to be given to staining. Beiderman *et al*., (1980) refer to the need to use a variety of staining techniques for chromosome identification.

Many of the methods utilized in the past for obtaining chromosomes from various avian species (some of which are listed in Appendix I) involve squash preparations and all have given poor results. In general, even though a wide variety of protocols have been followed, the chromosomes obtained are usually short, thus making it difficult to karyotype the species examined.

The lack of agreement on the taxonomy of the avian species based on chromosome analysis can be attributed to both the technical problems associated with the harvesting and staining and the relatively small amount of work that has been done on the karyotyping of the Charadriiformes (De Boer, 1984). Of the 300 or more species in the order Charadriiformes only about 20 have been karyotyped (Hammar 1966; Itoh *et al*., 1969; Hammar 1970; Ryttman *et al*., 1979 and Baker *et al*., 1981). Baker *et al*., (1981) karyotyped the Variable Oystercatcher (*Haematopus unicolor*) and found that its cells contained six more chromosomes than *Haematopus ostralegus* which had been karyotyped by Hammar (1970). He also found that *H. unicolor* had a submetacentric W chromosome whereas *H. ostralegus* had a telocentric W chromosome. Baker *et al*., (1981) state in

their paper that there is lack of certainty whether Hammar missed six B chromosomes or if the two species really possessed quite different diploid complements. However, Baker et al., (1981) and Hammar (1970) used different methodologies for demonstrating the chromosomes, and this may account for the six chromosome difference in the two species. Hammar's (1970) technique of squashing the tissue resulted in short, fat, poorly spread out chromosomes. The microchromosomes appeared as very small dots and it is quite possible that one or more could have been missed.

Hammar (1966; 1970) also karyotyped six species from the suborder Lari (L. canus, L. argentatus, L. ridibundus, S. paradisaea, S. hirundo and S. albifrons). Other species within the Charadriiformes studied by Hammar were V. vanellus, C. hiaticula, G. gallinago, N. arquata, T. totanus and R. avosetta.

Ryttman et al., (1979), did partial karyotypes of L. argentatus, L. fuscus, L. marinus and L. canus. The method used to obtain these karyotypes is briefly outlined (Appendix I). Ryttman et al., (1979) included only one chromosome of the nine largest chromosomes and the sex chromosomes in the partial karyotype and not the homologous pairs. These results showed that the Giemsa-banding pattern of the first 3 or 4 chromosomes of each species (L. argentatus, L. fuscus, L. marinus and L. canus) were similar.

Itoh et al., (1969) karyotyped L. argentatus. This

preparation was made before the introduction of the banding techniques, so the chromosome smears were stained with Giemsa stain. However, these workers discussed the chromosomes, using the terminology of the present study, but the criteria for determining what constituted a submetacentric, metacentric, subtelocentric, telocentric or acrocentric chromosome were not listed. Although they appeared to use the Levan nomenclature, they did not present the chromosome measurements or cite Levan in their remarks or references. They gave a description of the centromere position for the nine largest chromosomes. Chromosomes 1 and 2 are listed as being submetacentric although chromosome 2 is closer to being metacentric than submetacentric.

Since the early 1800's a number of techniques other than karyotyping have been used to group and classify the larids and alcids within and outside the order Charadriiformes. The larids have been thought to have close resemblances to the Procellariidae according to Nitzsch, 1840 (Sibley and Ahlquist, 1972) and the Columbidae (Gadow, 1889), while the alcids have been grouped with the Sphenisciformes (Coues, 1868), and with the Pelecanoidae (Verhegen, 1958), and the Gaviidae (Illiger, 1811; Verhegen, 1961).

Huxley (1867) grouped the larids and alcids together for the first time. They had also been grouped together by Garrod, 1873; Chandler, 1916 and Lowe, 1931. Wetmore

(1960) divided the Charadriiformes into three suborders, the Alcae (auks, murres and puffins), Lari (gulls, terns, jaegers and skimmers) and the Charadrii (the remaining groups). Sibley and Ahlquist (1972) compared the egg white proteins of non-passerine birds electrophoretically and concluded that the Alcidae were more closely related to the Laridae and other Charadriiformes than to the Gaviidae, Spheniscidae or the Pelecanoididae. There has been, then, considerable disagreement as to the true taxonomic position of these birds.

Besides the taxonomic problem of grouping the suborder Lari with Alcae, there are also questions as to whether *A. torda*, *U. aalge* and *F. arctic* should be within the same suborder. Gysels (1964) and Gysels and Rabaey (1964) examined the eye lens and muscle proteins of *U. aalge*, *A. torda* and *F. arctica* by zone electrophoresis and immunoelectrophoresis. The absence of glycogen in the lens indicated a close relationship between *Uria* and the Spheniscidae. Also *Alca* and *Fratercula* differed from the other Charadriiformes and from each other. In 1965 Averkina, *et al.*, conducted an immunological study of the serum proteins of the Alcidae and concluded that *Uria* and

Cepphus are closely related. *Alca* shows the next closest relation to these while *Fratercula* is more distant. Strauch (1985) analysed 33 cladistic characters of the skeleton, integument and natural history and concluded that *Alca* and *Uria* are closer to each other than

to *Fraterula*. From the results presented in this project the three karyotypes are distinct and it is difficult to determine a relationship between any two of the species. The karyotype of *A. torda* is different from that of *U. aalge* and *F. arctica* in that it has an acrocentric chromosome 3. The karyotype of *F. arctica* contains an acrocentric chromosome 11. Because the step from having an acrocentric chromosome to having a subtelocentric chromosome is not as great as going from an acrocentric chromosome to a submetacentric or metacentric chromosome, one would conclude that *U. aalge* and *A. torda* are closer to each other than to *F. arctica*.

Schnell (1970) using skeletal and external measurements determined that *L. argentatus* was closer to *L. marinus* than to *R. tridactyla*. Based on the differences in karyotypes presented herein *L. argentatus* appears to be closer to *R. tridactyla* than to *L. marinus* however further investigation is required.

Karyotyping may be useful in solving the taxonomic question, but before this may be done several problems have to be solved. First, a reliable method must be used to collect samples. Second, harvesting techniques, combined with adequate (and ideally, multiple) staining must be achieved.

SUMMARY

1. The embryos from the eggs of six seabird species, L. argentatus, L. marinus, R. tridactyla, U. aalge, A. torda and F. arctica, collected from Gull Island in the Witless Bay Seabird Sanctuary, were fragmented and cultured. The cells were subcultured from 1-8 times, then harvested for chromosomes.

2. Nineteen different harvesting procedures were attempted in order to find the procedure that yielded the best chromosome spreads. The method using 0.075 ng/ml of colcemid for 20 minutes at 37°C and the hypotonic sodium citrate at 21°C for 18 minutes gave the best results.

3. Attempts were made to stain the chromosomes using the Giemsa-banding technique, but failed. However, the Q-banding (fluorescent) technique worked well. Partial karyotypes were made from the chromosomes of the six seabird species.

4. The first 13 largest chromosomes (autosomes) were measured and the total length was taken to be 100%. Each segment (ie, p,q and c) was expressed as a ratio of the total length and the segment ratios were used to construct an idiogram. When more than one karyotype was made, the mean of each segment ratio was used. The three larid karyotypes were compared with each other and the differences among them were noted. Chromosome 10 in L. marinus is metacentric, different from the acrocentric 10

10 of L. argentatus and R. tridactyla. The L. argentatus karyotype differs from R. tridactyla in three chromosomes: 1) Chromosome 6 has a larger centromere in L. argentatus than in R. tridactyla, 2) Chromosome 8 is shorter in L. argentatus than in R. tridactyla; the p arm length is longer in R. tridactyla, and 3) Chromosome 9 is longer in L. argentatus than in R. tridactyla.

The differences in the chromosomes of the alcids are: 1) A. torda has an acrocentric chromosome 3 while U. aalge and F. arctica have a subtelocentric number three chromosome and 2) F. arctica has an acrocentric chromosome 11 while this chromosome is submetacentric in U. aalge and metacentric in A. torda.

5. The differences between the chromosomes of the larids and the alcids are: 1) the larids have an acrocentric chromosomes 5 and 11 while only F. arctica has an acrocentric 11 in the alcids examined and 2) chromosome 3 in the larids has a centromere. This chromosome does not have a centromere in U. aalge and F. arctica. A. torda also lacks a p arm for this chromosome.

6. In order to comment on the relationships of the three species (L. argentatus, L. marinus and R. tridactyla; U. aalge, A. torda and F. arctica) within the Laridae and Alcidae respectively, further work is required using more sophisticated methods for the harvesting and staining of the chromosomes and also employing other immunological and biochemical techniques.

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Appendix I. A review of the culturing, harvesting and staining methods used by other researchers and the species of birds examined.

AUTHOR(S)	YEAR	TISSUE	MEDIUM	HARVESTING	STAINING	SPECIES
Au, W., Fechheimer, N.S. & Soukup, S.	1975	Blood	McCoys 5a 30% fetal calf serum pokeweed mitogen (PWM)	10 ug/ml colcemide 3 h, buffy coat suspended in 1:3 bovine serum distilled water 10 min R.T., 1:3 acetic acid: methanol fixed	1. Centromere Bands 2. Giemsa or Orcein stain	Haliaeetus leucocephalus
Baker, A.J., Parslow, M. & Chambers, D.	1981	Embryo	Ham's F10 10% fetal calf serum	0.4 ug/ml colchicine 2 h, 0.075M KCl 37°C 20 min, 1:3 acetic acid & methanol fixed	1. Giemsa 2. Giemsa Bands 3. Centromere Bands 4. Silver stain	Haematopus unicolor
Biederman, B.M., Florence, O. & Lin, C.C.	1980	Blood	Ham's F10 5% chicken serum phytohaemagglutinin (PHA)	0.06 ug/ml colcemid 1 h, 0.075M KCl R.T. 18 min	1. Giemsa or aceto-orcein 2. Giemsa Banding 3. Q-Bands 4. C-Bands 5. Silver stain 6. Reverse Bands	Bubo virginianus
Bloom, S.E., Povar, G. & Peakall, D.B.	1972	Allantoic Sac	not cultured In Situ	0.02 ml 0.05% colcemid 45 min 37 - 38°C, squash preparation	1. Gram's Iodine	

AUTHOR(S)	YEAR	TISSUE	MEDIUM	HARVESTING	STAINING	SPECIES
Carlenius, C., Ryttman, H., Tegelstrom, H. & Jansson, H.	1981	Skin & Muscle from 10 day old foetuses	Eagles with Hepes 20% fetal calf serum	1 ug/ml colchicine 3 - 4 h, 0.075M KCl 10 - 15 min R.T., 3:1 methanol/ acetic acid fixed	1. G-Bands 2. C-Bands 3. R-Bands with Brdu	Gallus domesticus
DeBoer, L.E.M.	1976	Blood	Culture medium PWM	0.3 /ml colchicine 2 - 2 1/2 h, 0.075M KCl 5 min	1. Acetic orcein	Falconiformes (16 species)
DeBoer, L.E.M. & Belterman, R.H.R.	1981	Blood	PWM or PHA	1 x 10^{-4} % colchicine 1 1/2 h, 3:1 methanol /acetic acid	1. Acetic orcein 2. Q-Bands 3. Giemsa 4. C-Bands	Crax mitu
DeBoer, L.E.M. & Van Bocxstaele, R.	1981	Blood	PWM or PHA	1 x 10^{-4} % colchicine 1 1/2 h	1. Acetic orcein	Afropavo congensis
DeLucca, E.J.	1978	Spleen Liver Gonads	In situ	0.5 % colchicine injected, 50 % acetic acid fixed, squashed	1. Giemsa	Columbina picui Columbina minuta C.passerina C.talpacoti
Hammar, B.	1970	Embryonic		Pretreat 0.9 % NaCit & 0.1 % colchicine 20 - 30 min, alcohol acetic acid fixed, squashed	1. Acetic orcein	31 species

AUTHOR(S)	YEAR	TISSUE	MEDIUM	HARVESTING	STAINING	SPECIES
Hammar, B. & Herlin, M.	1975	Embryonic		Pretreat 0.9 % NaCit & 0.1 % colchicine 25 min 37^{0}C, squashed	1. Acetic orcein	4, Passeriformes (Motacillidae) (Fringillidae)
Hammar, B.	1966	Embryonic		Pretreat 0.9% NaCit & 0.1 % colchicine 25 min. 37°C alcohol-acetic acid 3:1, squashed	1. Orcein	9 species
Itoh, M., Ikeuchi, T., Shimba, H., Mori, M.,Susaki, M., & Makino, S.	1969	Feather pulp	TC 109 15% bovine serum	0.1 ug/ml colcemid 6 h, 0.075M KCl 15 min 37°C, 1:3 acetic acid/methanol	1. Giemsa stain	14 species including L.argentatus
Ray-Chaudhuri, R., Sharma, T. & Ray-Chaudhuri, S.P.	1969	Bone marrow	Eagles 15 % fetal calf serum PHA-M	colchicine slides hydrolyzed in warm 1N HCl & stained	1. Unna blue	11 species
Ryttman, H. & Tegel-strom, H.	1981	Skin Muscle	Eagles with Hepes 20% calf serum	1 ug/ml colchicine 6 - 8 h, 0.075M KCl 10 - 15 min, 3:1 methanol/acetic acid	1. G-Bands	3. Galliformes Gallus domesticus Gotumix japonica Meleagris galloporo
Ryttman, H., Tegel-strom, H. & Jansson, H.	1979	Foetal Skin Muscle	Eagles with Hepes 20% calf serum	1 ug/ml colchicine 3 h, 0.075M KCl 10 - 15 min, 3:1 methanol/acetic acid	1. G-Bands	Larus argentatus Larus fuscus Larus marinus Larus canus

AUTHOR(S)	YEAR	TISSUE	MEDIUM	HARVESTING	STAINING	SPECIES
Stock, A.D., Arrighi, F.E. & Stefos, K.	1974	Biopsy specimens from Aorta, Lung & Breast	McCoy's 5a 30% fetal calf serum	0.06 ug/ml colcemid 1 h, 3:1 H_2O: growth medium 10 min	1. G-Bands 2. C-Bands	*Columba livia domestica* *Streptopelia risoria* *Gallus domesticus*
Stock, A.D. & Bunch, T.D.	1982	Leg tissue nearly full term embryos	RPMI 1640 10% fetal calf serum	2 x 10^{-3} ug/ml colcemid 1 h 0.54% KCl 20 min 3:1 methanol: acetic acid	1. G-Bands 2. C-Bands	Galliformes 8 species
Stock, A.D. & Mengden G.	1975	Feather pulp	McCoy's 20% fetal calf serum		1. G-Bands	
Takagi, N. Itoh, M., & Sasaki, M.	1972	Blood also Feather pulp	Eagles MEM 20% fetal calf serum PHA	0.1 ug/ml colcemid & ^{3}H-thymidine (uC/ml) autoradiograph	1. Giemsa stain	4 Ratitae species
Takagi, N. & Sasaki, M.	1974	Blood	Eagles MEM 20% fetal calf serum PHA	0.1 ug/ml colcemid	1. G-Bands	48 species
Thorneycraft, H.B.	1975	Kidney Embryos Feather pulp Testes		Squash	1. Aceto-orcein	*Zonotrichia albicolis*

APPENDIX II

Reagents and Buffers.

1. Absolute Ethanol

2. Culture Medium

   2.1 Roswell Park Memorial Institute (RPMI) 1640 with 25mM Hepes Buffer and L-Glutamine from Grand Island Biological Company (Gibco), Grand Island, New York .... 500 ml

   2.2 Fetal Bovine Serum (Gibco) .... 100 ml

   2.3 Gentamycin (10 mg per ml) .... 2 ml

3. Hanks Basic Salt Solution without calcium or magnesium (Hanks BSS) (Gibco)

4. Colcemid (Gibco)
   Lyophilized rehydrated with 20 ml Hanks BSS to yield 5 ng per ml

5. Trypsin - Ethylenediaminetetra acetic acid (Trypsin-EDTA) 10 x Rehydrated with 20 ml of Hanks BSS (10 ml from two 100 ml bottles of Hanks BSS). Ten ml of this Trypsin-EDTA were added to the two bottles of Hanks BSS to give 2 x 100 ml of 1 x Trypsin-EDTA solution.

6. Potassium Chloride (75mM)
   5.4 g potassium chloride (KCl) Fisher Scientific Co., Halifax were dissolved in distilled water and made up to 1000 ml.

7. Sodium Citrate, 1.1%
   11.0 g sodium citrate (Fisher Scientific Co.) were dissolved in distilled water and made up to 1000 ml.

8. Fixative
   1 part glacial acetic acid
   3 parts absolute methanol

9. Fluorescent Stain
   9.1 Atebrin (Gurr, Searle Diagnostic, England)
   5 mg/50 ml distilled water
   9.2 0.02M sodium hydroxide (A)

9. 9.3 Tris-Maleic Buffer

24.2 g tris (Fisher) — dissolved in 1000 ml of distilled water (B)
23.2 g maleic Acid (Fisher)

Take 25 ml of (B) and pH to 5.6 with (A). Then make up to 100 ml with distilled water.

10. Giemsa Stain

10.1 Wright's stain (Sigma)
0.4 g Wright's per 100 ml absolute methanol
Stir at room temperature for 1 hour
Filter through No. 1 Whatman filter before use.

10.12 Buffer — made up to 1000 ml with
8.00 g sodium chloride
0.20 g potassium chloride
2.71 g sodium phosphate ($Na_2HPO_4.17H_2O$)
0.20 g potassium phosphate (KH_2PO_4)

11. Trypsin (Difco)

Appendix IIIA. Raw data for chromosome harvests.

DATE	SPECIES	NO. FLASKS HARVESTED	CULTURE TECHNIQUES	DAYS IN CULTURE	RESULTS	HARVEST NUMBER	METHOD LETTER
6/7	*L. argentatus* #1	2	0.05 ng/ml colcemid, 30 min. KCl 15 min. at 21° C	7	**	1,2	A
	L. argentatus #2	1	" " " " " " "	7	*	3	
	L. marinus #1	1	" " " " " " "	7	*	4	
7/7	*L. argentatus* #1	2	0.05 ng/ml colcemid, 10 min. KCl 15 min. at 21° C	8	**	5,6	B
	L. argentatus #2	3	0.075 ng/ml colcemid, 20 min. KCl 15 min. at 21° C	8	***	7,8,9	C
9/7	*L. argentatus* #1	2	0.05 ng/ml colcemid, 15 min. KCl 15 min. at 21° C	10	**	10,11	D
	R. tridactyla	1	0.05 ng/ml colcemid, 10 min. KCl 17 min. at 37° C	10	-	12	E
	U. aalge #2	1	" " " " " " "	5	-	13	
11/7	*L. argentatus* #2	2	0.05 ng/ml colcemid, 15 min. KCl 15 min. at 37° C	12	**	14,15	F
13/7	*L. marinus* #2	1	0.05 ng/ml colcemid, 5 min. KCl 17 min. at 37° C	14	-	16	G
	U. aalge #1	1	0.075 ng/ml colcemid, 15 min. KCl 17 min. at 37° C	9	-	17	H

+ = Good harvest, chromosomes not overlapping.
* = Poor harvest, chromosomes overlapping.
- = Harvest failure, no chromosomes in preparation.

Appendix IIIA. Raw data for chromosome harvests.

DATE	SPECIES	NO. FLASKS HARVESTED	CULTURE TECHNIQUES	DAYS IN CULTURE	RESULTS	HARVEST NUMBER	METHOD LETTER
16/7	L.marinus #1	2	0.075 ng/ml colcemid, 20 min. KCl 18 min. at 21° C	10	**	18,19	I
	F.arctica #1	4	0.05 ng/ml colcemid, 5 min. KCl 20 min. at 21° C	12	****	20-23	J
17/7	L.argentatus #1	2	0.075 ng/ml colcemid, 15 min. KCl 18 min. at 37° C	18	++	24,25	K
	L.argentatus #2	14	0.075 ng/ml colcemid, 15 min. KCl 17 min. at 37° C	18	+++**** --*****	26-39	H
	L.marinus #1	2	0.075 ng/ml colcemid, 30 min. KCl 18 min. at 21° C	18	**	40,41	L
	U.aalge #2	3	0.05 ng/ml colcemid, 15 min. KCl 20 min. at 21° C	13	***	42-44	M
18/7	L.marinus #2	1	0.075 ng/ml colcemid, 5 min. KCl 20 min. at 37° C	19	-	45	N
	R.tridactyla	6	0.05 ng/ml colcemid, 5 min. KCl 20 min. at 21° C	19	*** ***	46-51	J
	F.arctica #1	1	0.075 ng/ml colcemid, 15 min. KCl 20 min. at 21° C	14	+	52	O
	F.arctica #2	2	" " " " " " "	14	*-	53,54	

+ = Good harvest, chromosomes not overlapping.
* = Poor harvest, chromosomes overlapping.
- = Harvest failure, no chromosomes in preparation.

Appendix IIIA. Raw date for chromosome harvests.

DATE	SPECIES	NO. FLASKS HARVESTED	CULTURE TECHNIQUES	DAYS IN CULTURE	RESULTS	HARVEST NUMBER	METHOD LETTER
18/7	U.aalge #1	6	0.075 ng/ml colcemid, 5 min. KCl 20 min. at 21° C	14	--* ***	55-60	P
23/7	F.arctica #2	1	0.075 ng/ml colcemid, 5 min. KCl 20 min. at 21° C	19	-	61	P
24/7	R.tridactyla	2	0.075 ng/ml colcemid, 20 min. NaCit. 18 min. at 21° C	20	+*	62,63	Q
25/7	F. arctica #2	1	0.075 ng/ml colcemid, 20 min. NaCit. 15 min. at 21° C	21	*	64	R
	A.torda	1	" " " " " " "	20	-	65	
26/7	L.marinus #2	1	0.075 ng/ml colcemid, 20 min. NaCit. 18 min. at 21° C	27	*	66	Q
	F.arctica #2	3	" " " " " " "	22	++*	67-69	
	A.torda	2	" " " " " " "	21	++	70,71	

+ = Good harvest, chromosomes not overlapping.
* = Poor harvest, chromosomes overlapping.
- = Harvest failure, chromosomes in preparation.

Appendix IIIA. Raw data for chromosome harvests.

DATE	SPECIES	NO. FLASKS HARVESTED	CULTURE TECHNIQUES	DAYS IN CULTURE	RESULTS	HARVEST NUMBER	METHOD LETTER
28/7	F. arctica #1	1	0.075 ng/ml colcemid, 20 min. NaCit. 18 min. at 21° C	24	+	72	Q
	R. tridactyla	2	" " " " " " "	24	**	73,74	
29/7	R. tridactyla	1	0.075 ng/ml colcemid, 20 min. NaCit. 18 min. 21° C	25	+	75	Q
	F. arctica #1	1	" " " " " "	25	*	76	
	U. aalge #2	1	" " " " " "	25	+	77	
	U. aalge #1	3	0.075 ng/ml colcemid, 15 min. NaCit. 18 min. at 21° C	25	---	78-80	S
30/7	L. argentatus #1	2	0.075 ng/ml colcemid, 20 min. NaCit 18 min. at 21° C	31	++	81,82	Q
	L. argentatus #2	2	" " " " " " "	31	+*	83,84	
	L. marinus #1	1	" " " " " " "	31	*	85	
	R. tridactyla	2	" " " " " " "	26	++	86,87	
	U. aalge #1	1	0.075 ng/ml colcemid, 15 min. NaCit. 18 min. at 21° C	21	-	88	S

+ = Good harvest, chromosomes not overlapping.
* = Poor harvest, chromosomes overlapping.
- = Harvest failure, no chromosomes in preparation.

Appendix IIIA. Raw data for chromosome harvest.

DATE	SPECIES	NO. FLASKS HARVESTED	CULTURE TECHNIQUES	DAYS IN CULTURE	RESULTS	HARVEST NUMBER	METHOD
4/8	F.arctica #1	1	0.075 ng/ml colcemid, 20 min. NaCit. 18 min. at 21 C	31	-	89	Q
5/8	L.argentatus #1	1	" " " " " " "	37	*	90	
6/8	L.argentatus #1	2	" " " " " " "	38	**	91,92	
	L.argentatus #2	2	" " " " " " "	38	--	93,94	
	L.marinus #1	2	" " " " " " "	38	--	95,96	
7/8	L.argentatus #1	1	" " " " " " "	39	-	97	
	L.argentatus #2	2	" " " " " " "	39	++	98,99	
	L.marinus #1	2	" " " " " " "	39	++	100,101	
8/8	R.tridactyla	2	" " " " " " "	35	*-	102,103	
11/8	L.argentatus #1	2	" " " " " " "	43	--	104,105	
	L.argentatus #2	2	" " " " " " "	43	++	106,107	
	L.marinus #1	2	" " " " " " "	43	++	108,109	
12/8	L.marinus #2	1	" " " " " " "	44	-	110	
	F.arctica #2	1	" " " " " " "	39	-	111	
	U.aalge #2	1	" " " " " " "	39	+	112	
	A.torda	1	" " " " " " "	38	*	113	

+ = Good harvest, chromosomes not overlapping.
* = Poor harvest, chromosomes overlapping.
- = Harvest failure, no chromosomes in preparation.

Appendix IIIA. Raw data for chromosome harvest.

DATE	SPECIES	NO. FLASKS HARVESTED	CULTURE TECHNIQUES	DAYS IN CULTURE	RESULTS	HARVEST NUMBER	METHOD LETTER
14/8	L.argentatus #2	2	0.075 ng/ml colcemid, 20 min. NaCit. 18 min. at 21° C	46	++	114,115	Q
	L.marinus #2	1	" " " " " " "	46	-	116	
15/8	L.marinus #1	2	" " " " " " "	47	++	117,118	
	U.aalge #1	2	" " " " " " "	42	++	119,120	
20/8	L.marinus #2	1	" " " " " " "	52	+	121	
	U.aalge #2	1	" " " " " " "	47	-	122	
	A.torda	1	" " " " " " "	46	-	123	
21/8	U.aalge #2	1	" " " " " " "	48	+	124	
22/8	L.marinus #1	2	" " " " " " "	54	++	125,126	
	U.aalge #1	1	" " " " " " "	49	-	127	
25/8	L.marinus #1	2	" " " " " " "	57	++	128,129	
	U.aalge #1	1	" " " " " " "	52	+	130	
26/8	F.arctica #2	1	" " " " " " "	53	+	131	
27/8	L.marinus #2	1	" " " " " " "	59	-	132	

+ = Good harvest, chromosomes not overlapping.
* = Poor harvest, chromosomes overlapping.
- = Harvest failure, no chromosomes in preparation.

Appendix IIIB. A summary of the Q method harvests for the Larids.

DATE	HARVEST NUMBER	SPECIES	DAYS IN CULTURE	TOTAL NUMBER OF HARVESTS	GRADINGS
30/7	94,95	L.argentatus #1	31	2	++
5/8	103		37	1	*
6/8	104,105		38	2	**
7/8	110		39	1	-
11/8	117,118		43	2	--
30/7	96,97	L.argentatus #2	31	2	+*
6/8	106,105		38	2	--
7/8	111,112		39	2	++
11/8	119,120		43	2	++
14/8	127,128		46	2	++
30/7	98	L.marinus #1	31	1	*
6/8	108,109		38	2	--
7/8	113,114		39	2	++
11/8	121,122		43	2	++
15/8	130,131		47	2	++
22/8	138,139		54	2	++
25/8	142,143		57	2	++
26/7	72	L.marinus #2	27	1	*
12/8	123		44	1	-
14/8	129		46	1	-
20/8	134		52	1	+
27/8	146		59	1	-
24/7	68,69	R.tridactyla	20	2	+*
28/7	85,86		24	2	**
29/7	87		25	1	+
30/7	99,100		26	2	++
8/8	115,116		35	2	*-

+ = GOOD HARVEST
* = POOR HARVEST
- = HARVEST FAILURE

Appendix IIIB. A summary of the Q method harvests for the Alcids.

DATE	HARVEST NUMBER	SPECIES	DAYS IN CULTURE	TOTAL NUMBER OF HARVESTS	GRADINGS
15/8	132,133	U.aalge #1	42	2	++
22/8	141		49	1	-
25/8	144		52	1	+
29/7	89	U.aalge #2	25	1	+
12/8	125		39	1	+
20/8	135		47	1	-
21/8	137		48	1	+
26/7	76,77	A.torda	21	2	++
12/8	126		38	1	*
20/8	136		46	1	-
28/7	78	F.arctica #1	24	1	+
29/7	88		25	1	*
4/8	102		31	1	-
26/7	73,74 75	F.arctica #2	22	3	++*
12/8	124		39	1	-
26/8	145		53	1	+

+ = GOOD HARVEST

* = POOR HARVEST

- = HARVEST FAILURE

Appendix IVA. The size of the p, c and q portions expressed as a percent of the total length of the haploid genome for the Larids.

		L. argentatus			*L. marinus*	*R. tridactyla*
		1	2	X	1	1
	p	7.0	7.4	7.2	5.7	6.8
1	c		0.6	0.3	0.6	0.5
	q	12.4	13.0	12.7	13.7	13.2
	p	6.0	6.2	6.1	5.7	5.9
2	c	0.5	0.3	0.4	0.6	0.5
	q	10.0	9.6	9.8	9.1	9.5
	p	1.0	1.1	1.1	0.9	0.9
3	c	0.2	0.3	0.3	0.6	0.2
	q	10.9	10.2	10.6	1.2	11.8
	p	2.0	2.0	2.0	1.7	2.3
4	c	0.7	0.8	0.8	1.1	0.5
	q	7.5	6.2	6.9	6.3	6.8
	p					
5	c					
	q	8.5	7.4	7.9	8.0	7.7
	p	1.0	1.1	1.1	1.1	1.1
6	c	0.7	0.6	0.7	0.3	0.2
	q	6.0	5.1	5.6	5.1	5.5
	p	1.0	1.4	1.2	1.1	1.8
7	c	0.7	0.6	0.7	0.6	0.5
	q	5.0	4.5	4.8	3.4	4.1
	p	1.3	1.7	1.5	2.3	1.8
8	c	0.5	0.6	0.6	0.9	0.5
	q	4.0	3.4	3.7	2.3	4.1
	p	2.0	2.3	2.2	1.7	1.8
9	c	0.5	0.8	0.7	0.6	0.2
	q	2.5	2.8	2.7	2.3	2.3
	p				1.7	
10	c				0.3	
	q	3.0	3.4	3.2	1.7	3.2
	p					
11	c					
	q	2.0	2.3	2.2	3.4	2.3
	p					
12	c					
	q	2.0	2.3	2.2	2.3	2.3
	p					
13	c					
	q	1.5	2.0	1.8	2.3	1.0

Appendix IVB. The size of the p, c and q portions expressed as a percent of the total length of the haploid genome for the Alcids.

		U. aalge			*A. torda*			*F. artica*			
		1	2	X	1	2	X	1	2	3	X
1	p	6.8	7.6	7.2	7.7	7.3	7.5	7.5	8.4	7.9	7.9
	c	0.8	0.9	0.9	0.8	0.9	0.9	0.9	1.0	1.2	1.0
	q	14.5	16.1	15.3	13.8	13.4	13.6	13.7	13.0	14.5	13.7
2	p	6.2	5.7	6.0	5.5	6.1	5.8	5.6	5.8	6.1	5.8
	c	0.8	0.5	0.7	0.8	0.6	0.7	0.9	1.0	1.2	1.0
	q	9.9	8.1	9.0	8.8	10.3	10.0	11.2	11.7	9.1	10.7
3	p	1.0	1.4	1.2				1.2	1.0	1.2	1.1
	c										
	q	10.9	10.4	10.7	11.6	12.2	11.9	11.2	11.7	11.5	11.5
4	p	2.1	1.9	2.0	2.2	1.8	2.0	1.2	1.3	1.5	1.3
	c	0.8	0.7	0.8	0.6	0.6	0.6	0.6	0.6	0.6	0.6
	q	5.7	6.6	6.2	6.1	6.1	6.1	6.2	6.5	6.1	6.3
5	p	2.1	1.4	1.8	1.7	1.8	1.8	1.2	1.0	0.6	0.9
	c	0.8	0.7	0.8	0.6	0.6	0.6	0.3	0.3	0.3	0.3
	q	5.7	6.6	6.2	4.4	4.9	4.7	5.6	5.2	6.1	5.6
6	p	2.1	1.9	2.0	2.2	1.8	2.0	1.5	1.3	1.8	1.5
	c	1.0	0.9	1.0	0.8	0.6	0.7	1.2	1.3	1.2	1.2
	q	2.6	2.8	2.7	3.0	2.5	2.7	2.5	2.6	2.4	2.5
7	p	2.1	1.9	2.0	2.2	2.1	2.2	1.9	1.6	1.8	1.8
	c	0.5	0.5	0.5	0.8	0.9	0.9	0.9	0.6	0.9	0.8
	q	2.1	1.9	2.0	2.8	2.1	2.5	2.5	2.6	1.8	2.3
8	p	1.6	1.9	1.8	2.2	1.8	2.0	1.9	1.9	1.8	1.9
	c	0.5	0.5	0.5	0.8	0.9	0.9	0.6	0.6	0.9	0.7
	q	2.1	2.4	2.3	2.2	2.1	2.2	2.5	2.6	1.8	2.3
9	p	1.6	0.9	1.3	1.9	1.8	1.9	1.2	1.3	1.5	1.3
	c	0.5	0.5	0.5	0.6	0.6	0.6	0.3	0.3	0.6	0.4
	q	2.6	2.8	2.7	1.9	1.8	1.9	1.9	1.6	1.8	1.8
10	p	1.6	0.9	1.3	1.7	1.2	1.5	1.2	1.3	1.5	1.3
	c	0.3	0.5	0.4	0.6	0.6	0.6	0.6	0.6	0.6	0.6
	q	2.1	2.4	2.3	1.7	1.5	1.6	1.2	1.3	1.8	1.4
11	p	1.0	0.9	1.0	1.7	1.2	1.5				
	c				0.6	0.6	0.6				
	q	2.6	2.8	2.7	1.7	2.4	2.1	3.1	3.9	3.6	3.5
12	p										
	c										
	q	3.1	2.4	2.8	3.3	3.6	3.5	3.7	3.2	3.0	3.3
13	p										
	c										
	q	2.1	2.4	2.3	2.8	3.0	2.9	2.8	2.8	3.0	2.8

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