Female specific markers and attempts of all-female production in half-smooth tongue sole (*Cynoglossus semilaevis*)

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ISGA XII, Spain, 2015.06.23

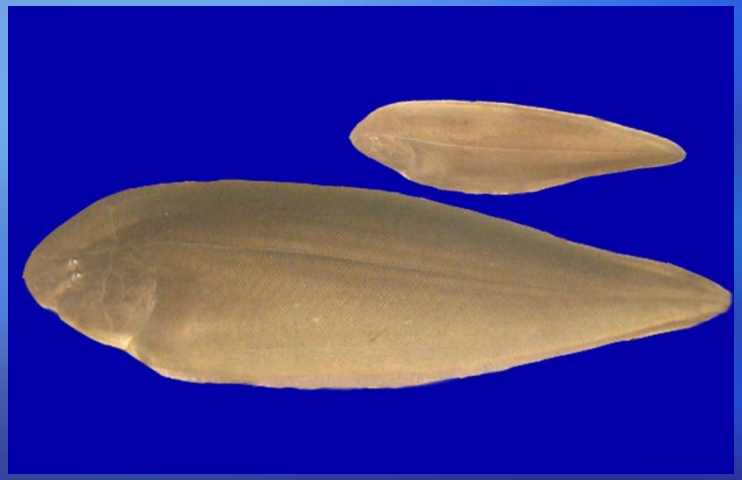
Why sex control in fish

1、In many species, growth difference between sexes

2、Control unexpected reproduction and population size

3、Some species have different maturation age

Sexual dimorphism in half-smooth tongue sole

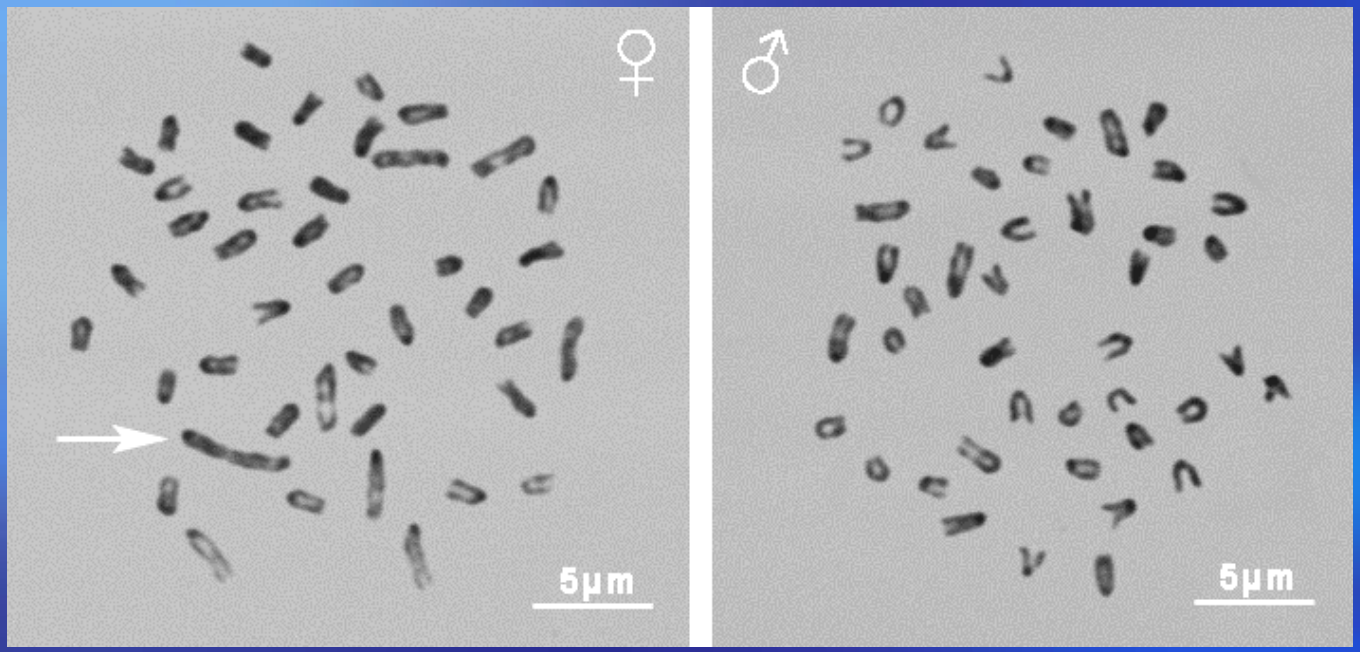


***Cynoglossus semilaevis*,**

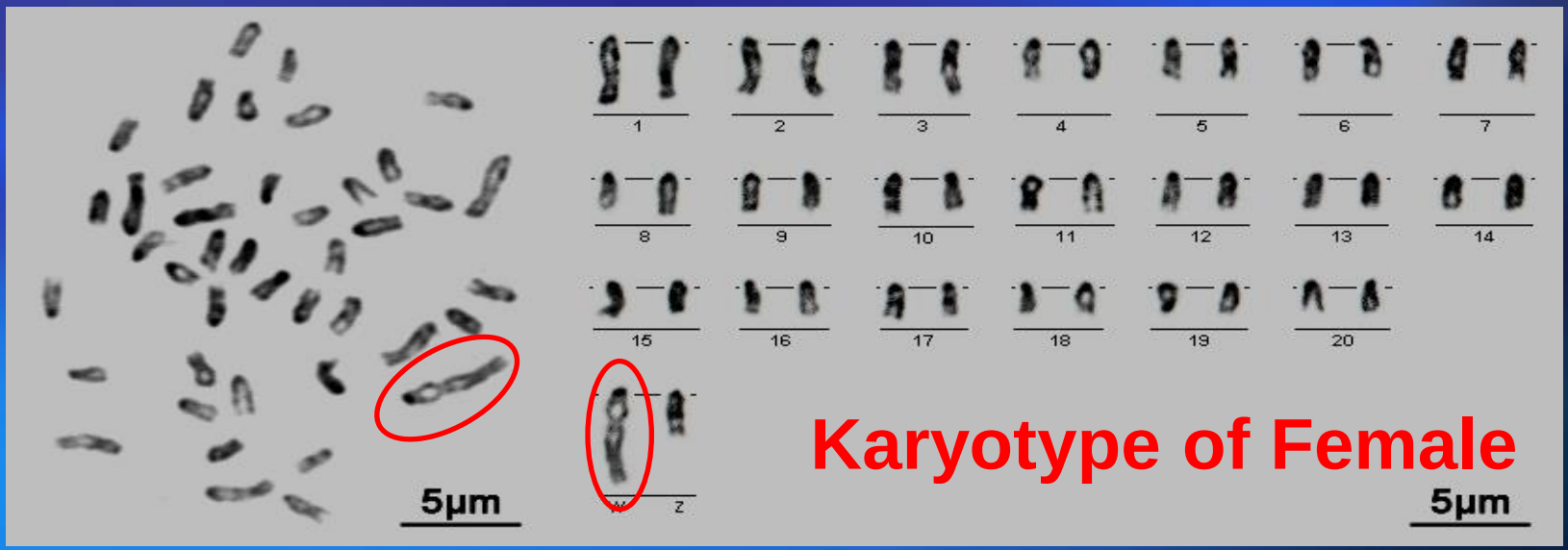
(Pleuronectiformes, Cynoglossidae)

Important aquaculture flatfish showing significant fast growth and larger size in female than in male

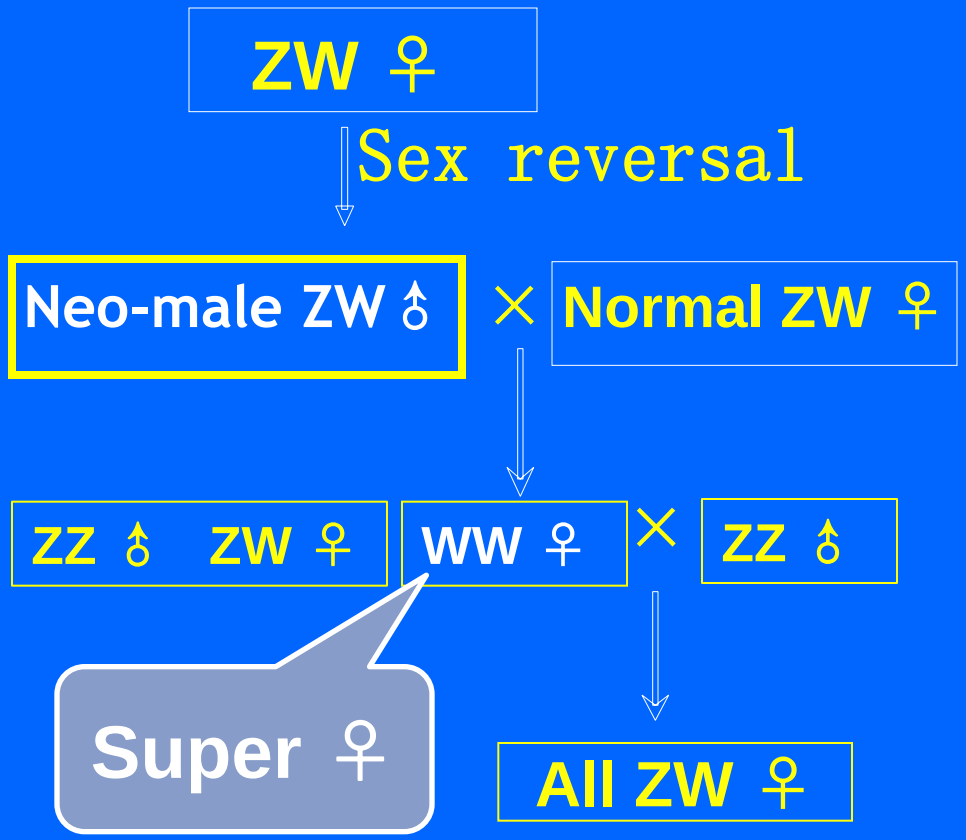
Sex determination system

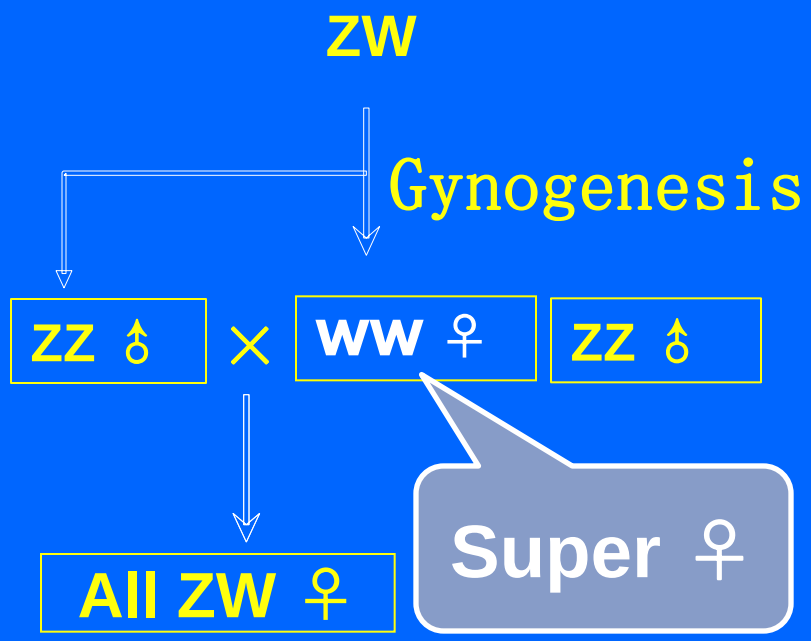


ZW/ZZ



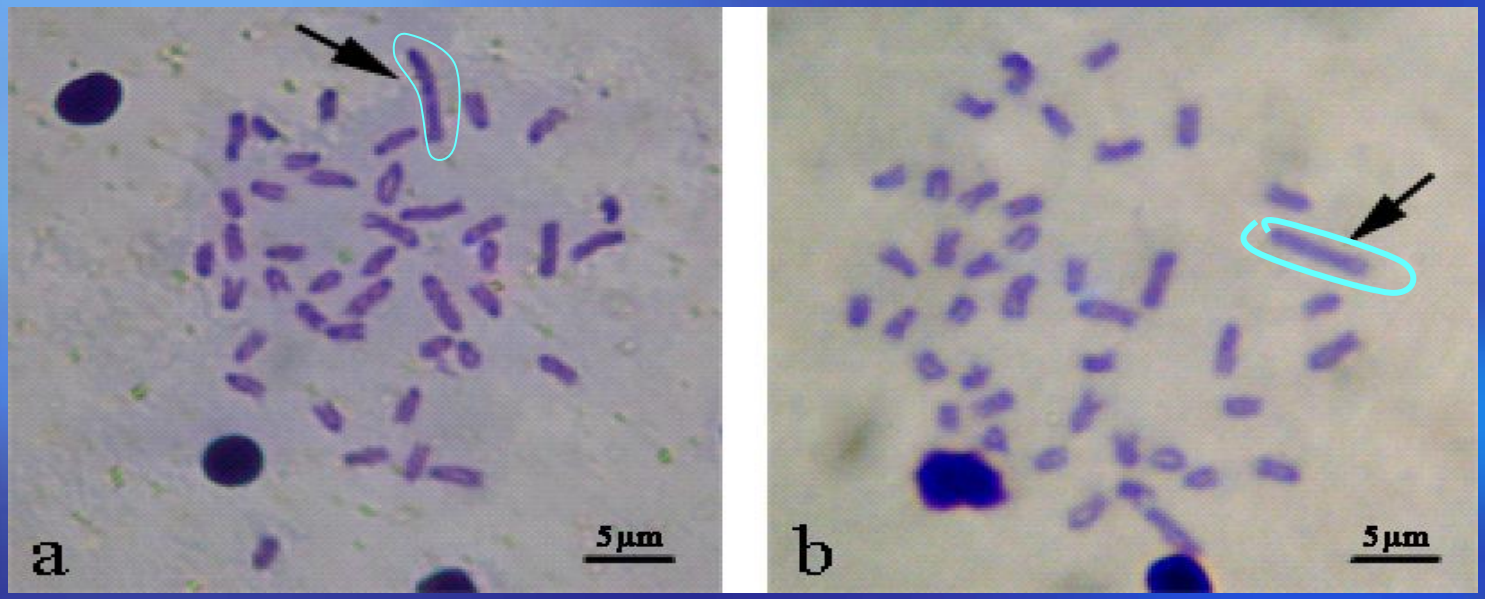
Ways for producing all female offspring





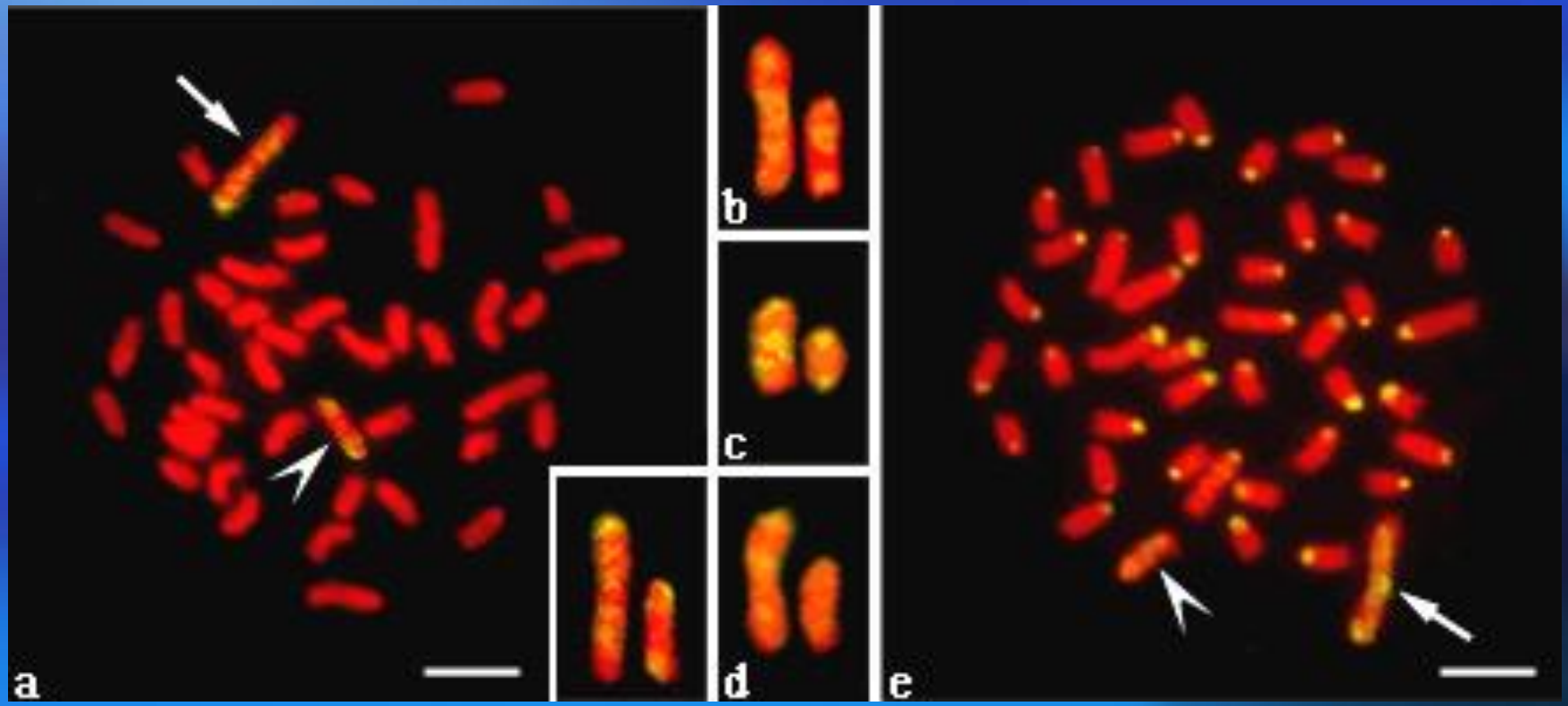
Q: How to Identify ZW Neo-male from normal ZZ male?
How to identify WW super female from normal ZW female?

W chromosome microdisection



- A total of 100 W chromosomes were dissected
- DOP PCR amplification of dissected W—DNA
- W specific library was constructed
- W specific markers were developed
- Molecular sexing method was developed

With DOP-PCR amplified W--DNA as probe, reverse painting was conducted
Z and W chromosomes were recognized.

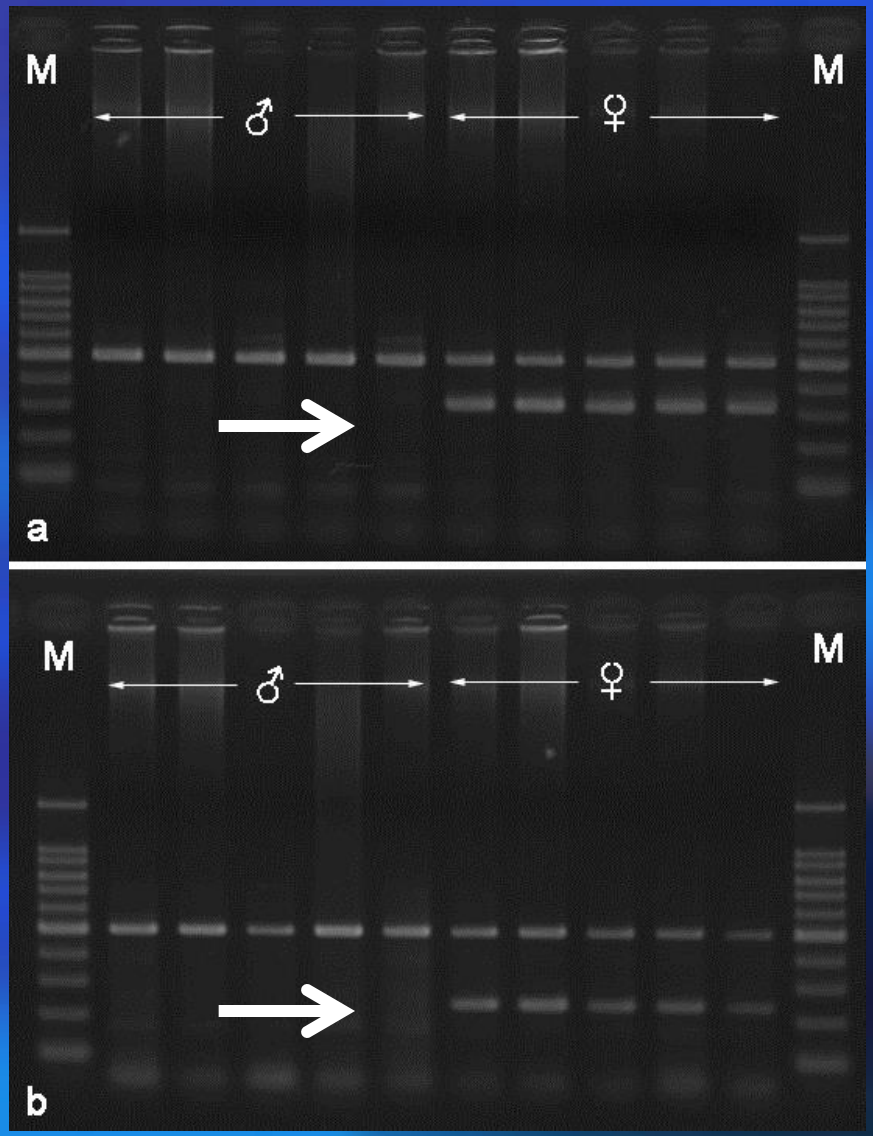


Aquaculture 297, 78–84

Molecular Sexing

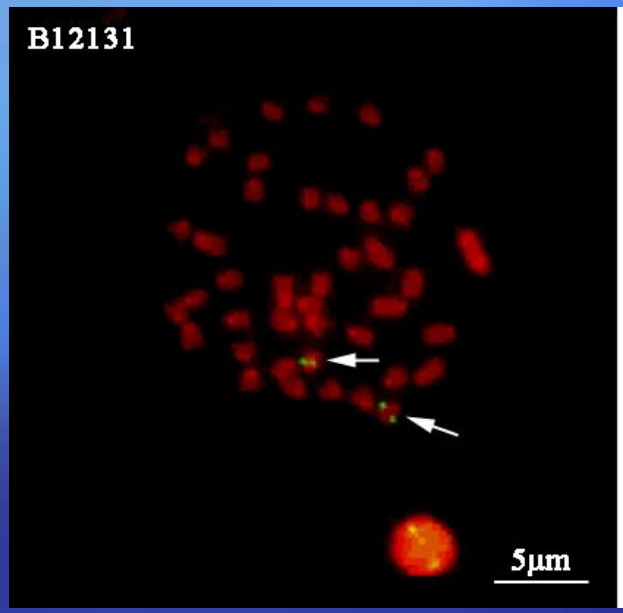
From the W specific library, several female specific markers were identified.

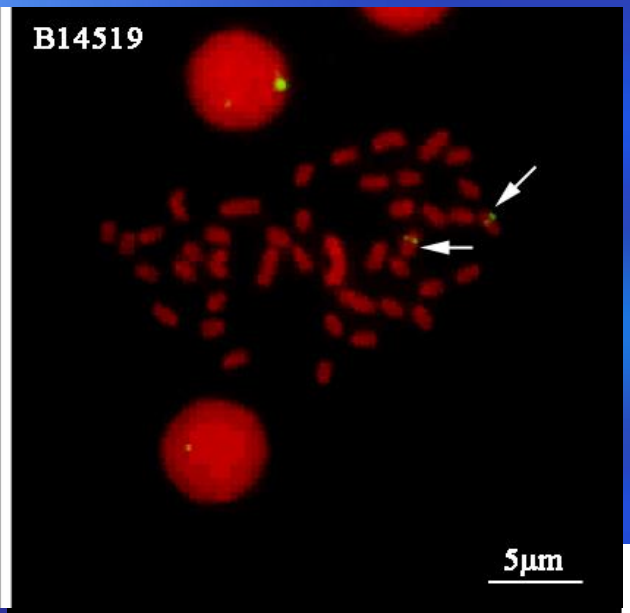
By simple PCR, the genetic sexes can be easily distinguished



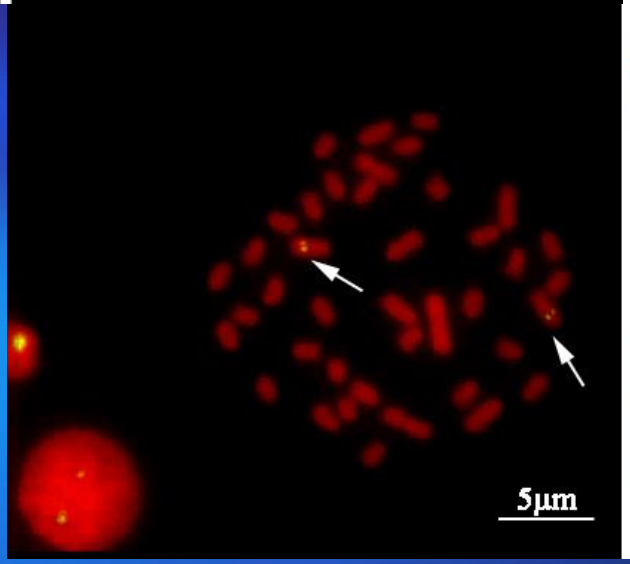
Aquaculture 388–391 (2013) 49–53

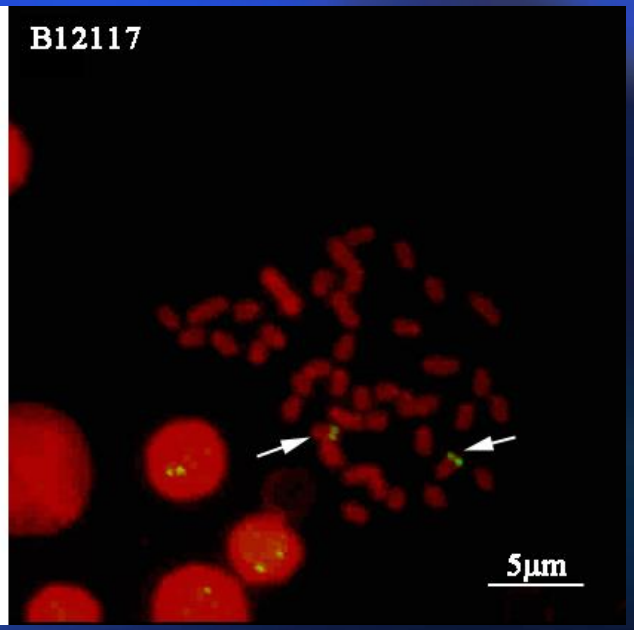
We also constructed Fosmid library with the average insert size of 30Kb



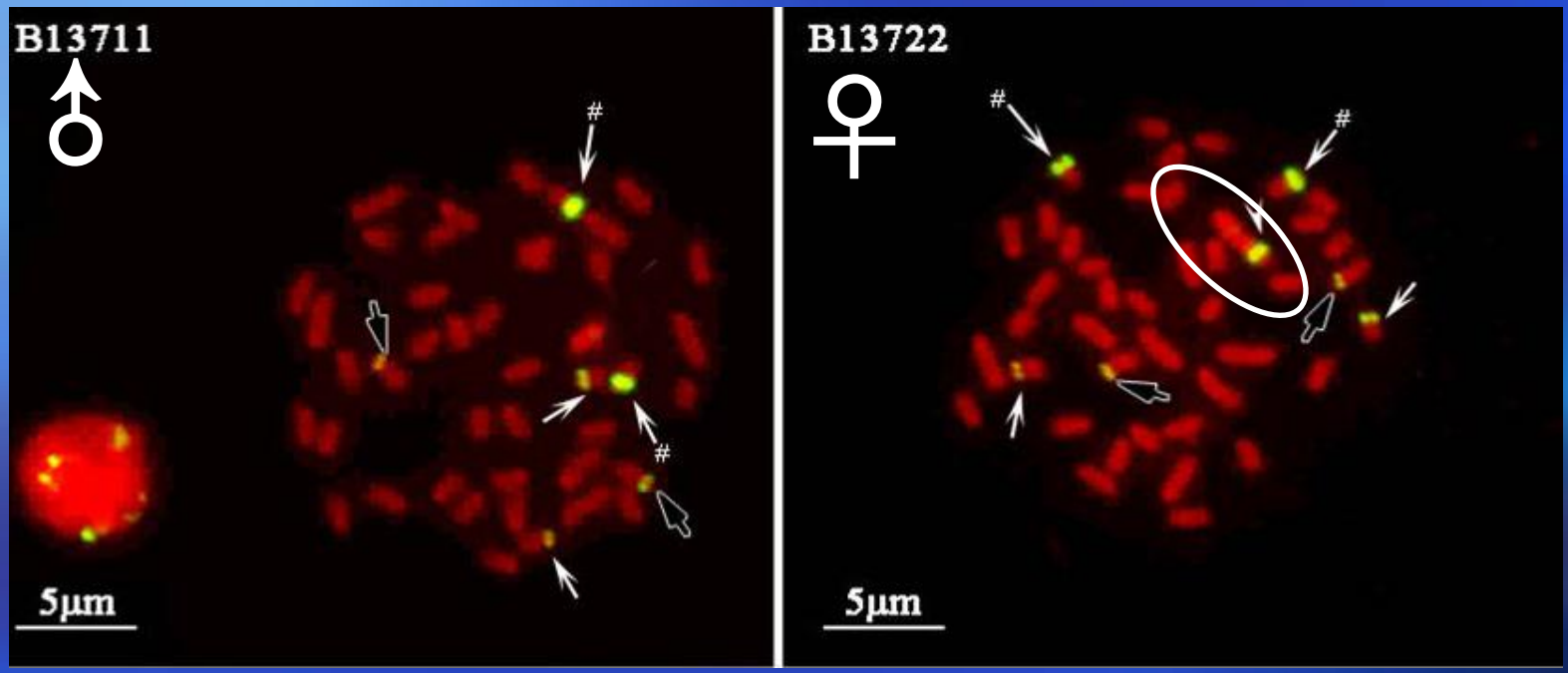


FISH analysis showed that most F clones give one pair of signals





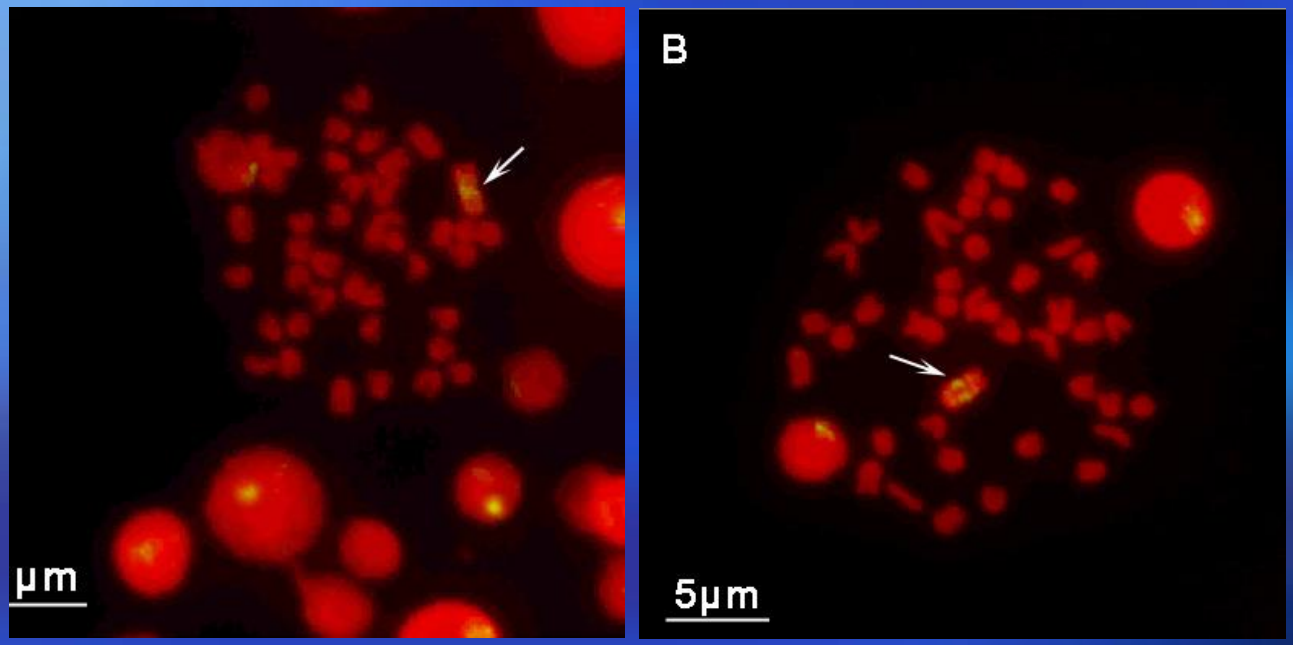
Identification of W-recognizing Fosmid clone



When using rDNA containing clone as probe

- **male cells showed 6 signals**
- **female cells showed 7 signals, with one large signal on W chromosome**

W specific markers ➡ screen of W specific fosmid clones ➡ FISH



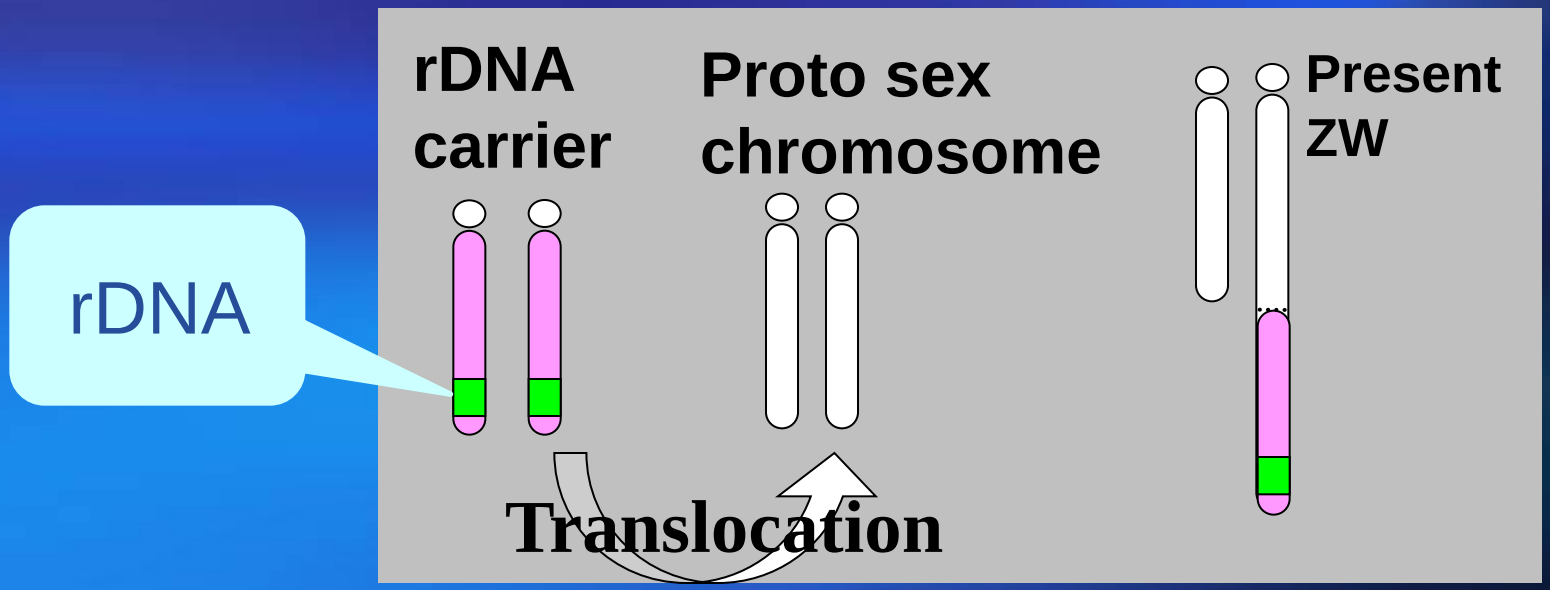
Several W specific fosmid clones were identified--mapped only on W chromosome

They can be used to distinguish WW from ZW

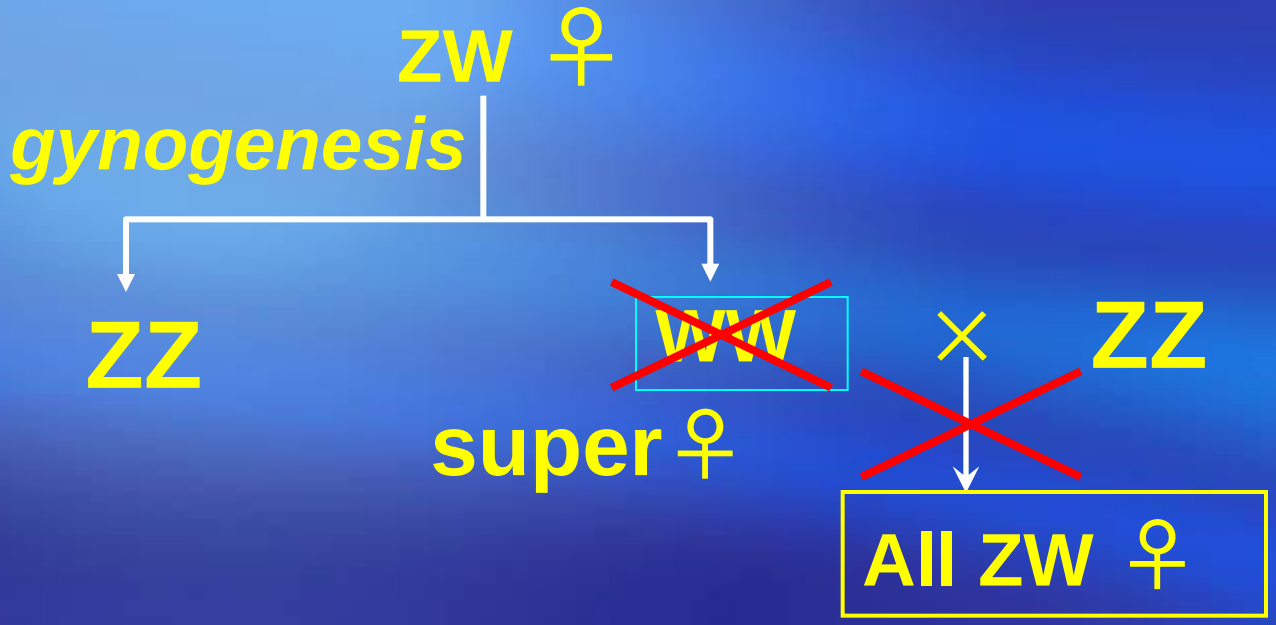
Possible origin of the W chromosome

The existence of obvious sex chromosomes in Pleuronectiformid fish is rare. The formation of extremely large W chromosome in half-smooth tongue sole may be a relatively “recent” event

We guess: One rDNA carrying chromosome translocated to the Proto-sex chromosome



All female production attempt 1: gynogenesis



ZZ	♂	ZZ	♂
ZZ	♂	ZZ	♂
ZZ	♂	ZZ	♂
ZZ	♂	ZZ	♂
ZZ	♂	ZZ	♂
ZZ	♂	ZZ	♂
ZZ	♂	ZZ	♂
ZZ	♂	ZZ	♂
ZZ	♂	ZZ	♂
ZZ	♂	ZZ	♂
ZZ	♂	ZZ	♂
ZZ	♂	ZZ	♂

Chromosomes for 200 gyogens, all individuals were ZZ male，

PCR assay did not find W markers in the offspring.

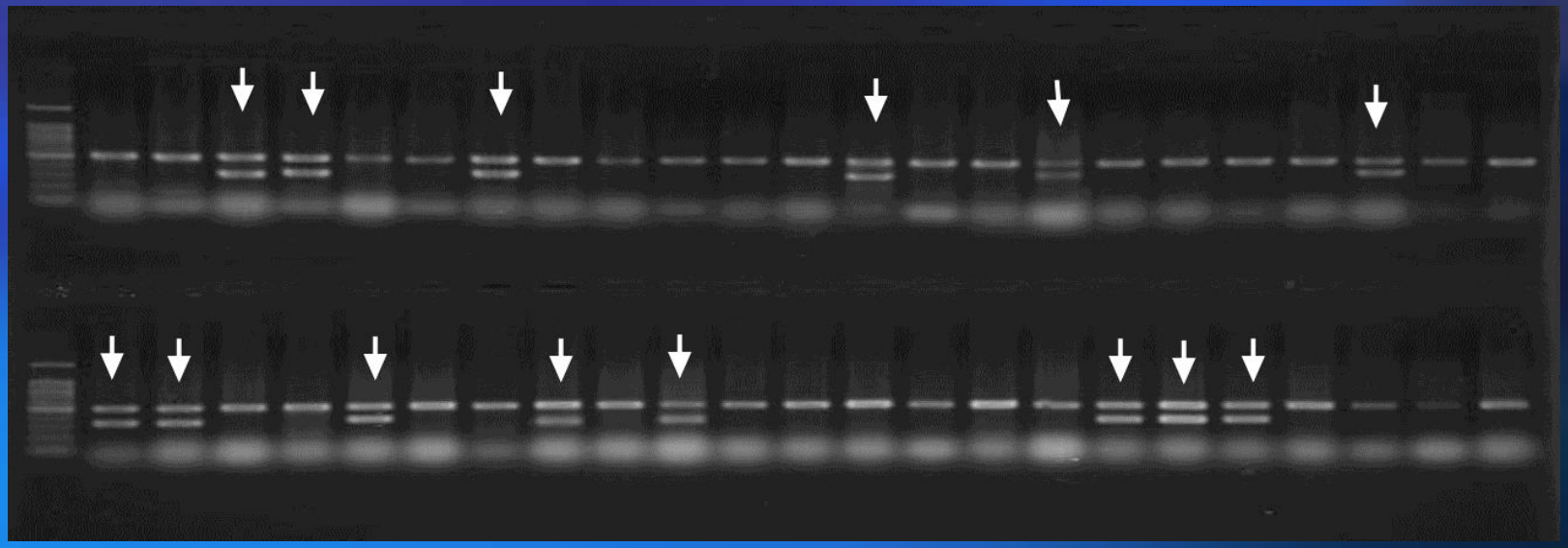
Gynogenesis cannot obtain WW!

All female production attempt 2： sex reversal

Among the sex reversed stock, more than 70% individuals were physiological males.

PCR assay identified 34% of the males were genetically ZW pseudo-male.

More than 800 ZW neo-males were screened

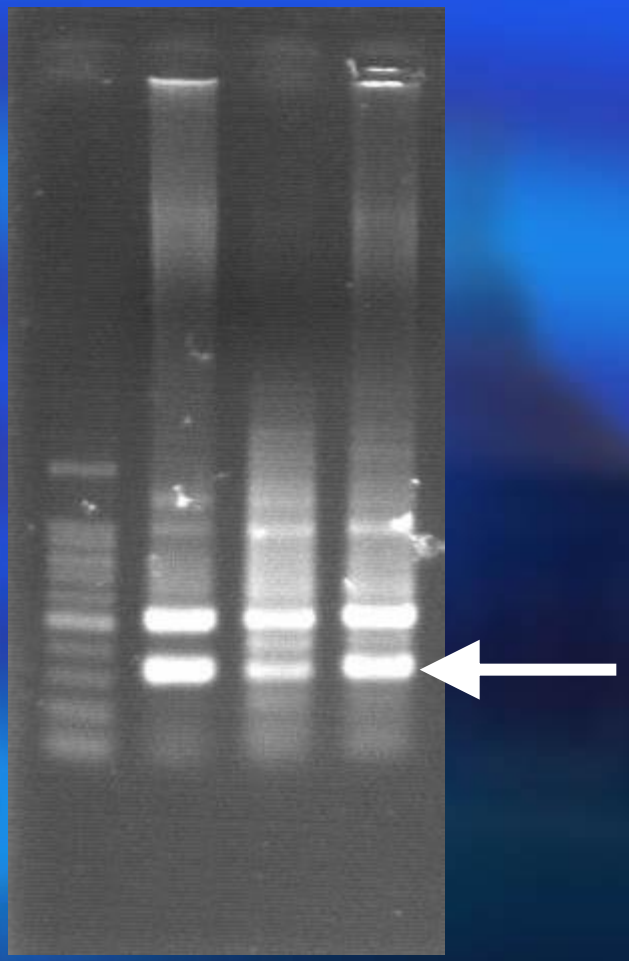


Most ZW males got mature and produced sperms

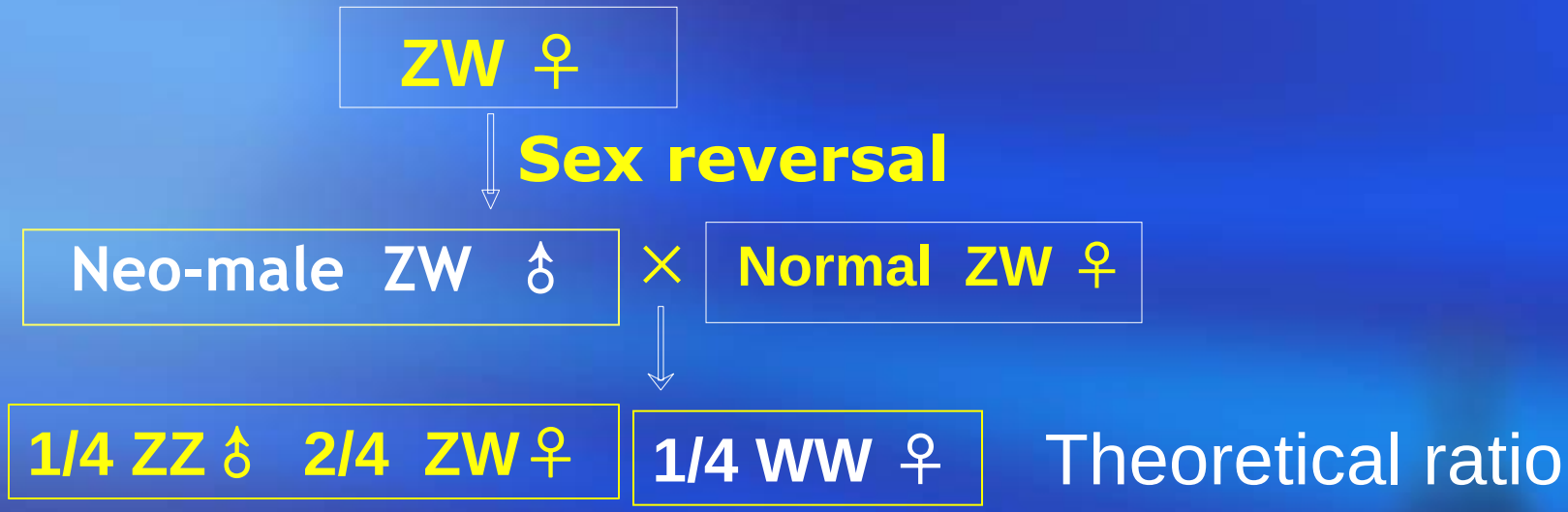
PCR detection can find the W specific marker from all sperms produced by ZW males。

This means:

W sperms were produced



All female production attempt 2: using ZW males



Among the offspring of ZW male,

about 60% individuals have W specific marker.

Chromosomes for more than 200 individuals revealed that all these were genetically ZW.

WW super female was not found

All female production attempt: using ZW male

ZW ♀

Artificial sex reversal

Neo-male ZW ♂ × Normal ZW ♀

1/4 ZZ ♂ 2/4 ZW ♀

60% of females can be obtained

Can we use this directly?

How would be the growth performance of these fish??

~~1/4 WW ♀~~ × ZZ ♂

All ZW ♀

Growth of offspring from ZW X ZW cross

	ZW X ZW		Control ZW X ZZ	
	♀	♂	♀	♂
Total No	67	58	33	45
Sex ratio	59.6	40.4	42.3	57.4
Max. BL	24.5	17.4	25.4	19.3
Min. BL	10.7	9.6	14.5	11.8
Av. BL	17.3	16.0	22.2	17.6
Max. BW	98.0	25.2	110.3	36.6
Min. BW	6.6	4.7	12.7	8.9
Av. BW	24.5	15.0	35.8	16.5

The contribution of W sperms was not the same to that of W eggs

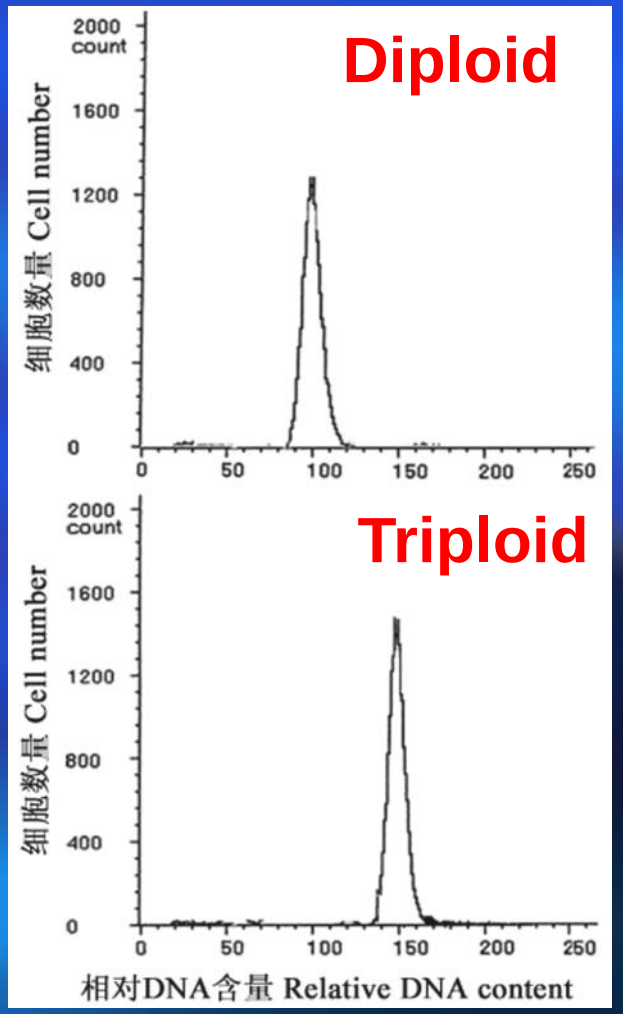
Alternative methods?— polyploidy?

ZW ♀ × ZZ ♂ **Normal cross**

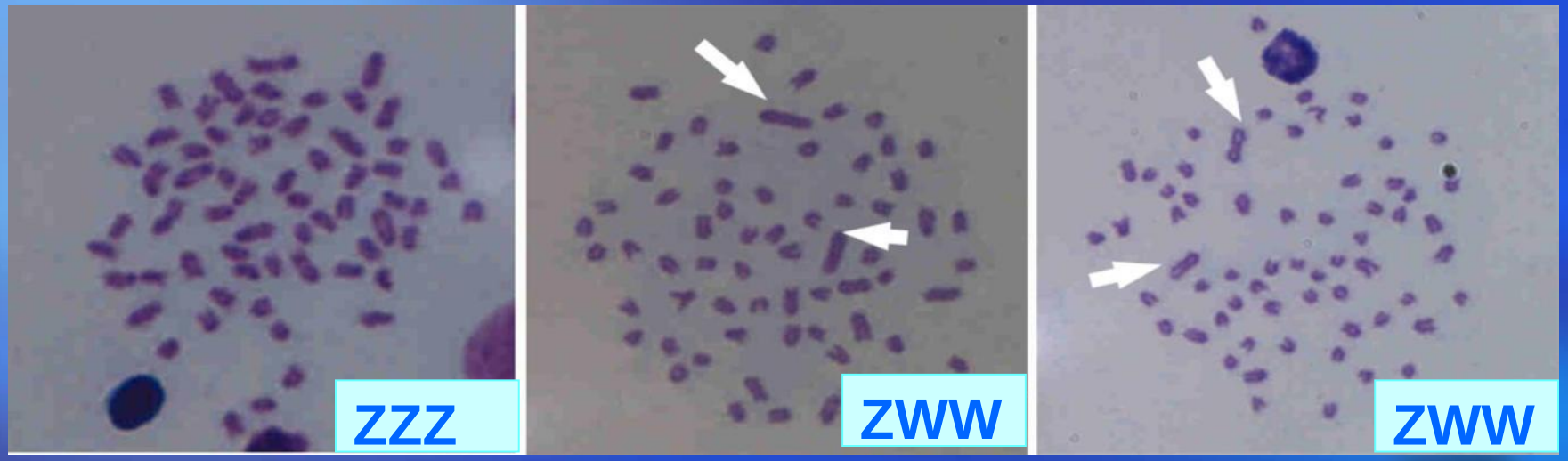
Suppression of the PB2 release in the fertilized eggs

ZWW　ZZZ　ZZW? ?

In the induced group more than 80% were triploids



Chromosomes of triploids



Chromosomes were observed in 30 individuals at average body length of 15cm

- ✓ **ZZZ 16**
- ✓ **ZWW 14**
- ✓ **ZZW 0**

Sex and growth performance of triploids

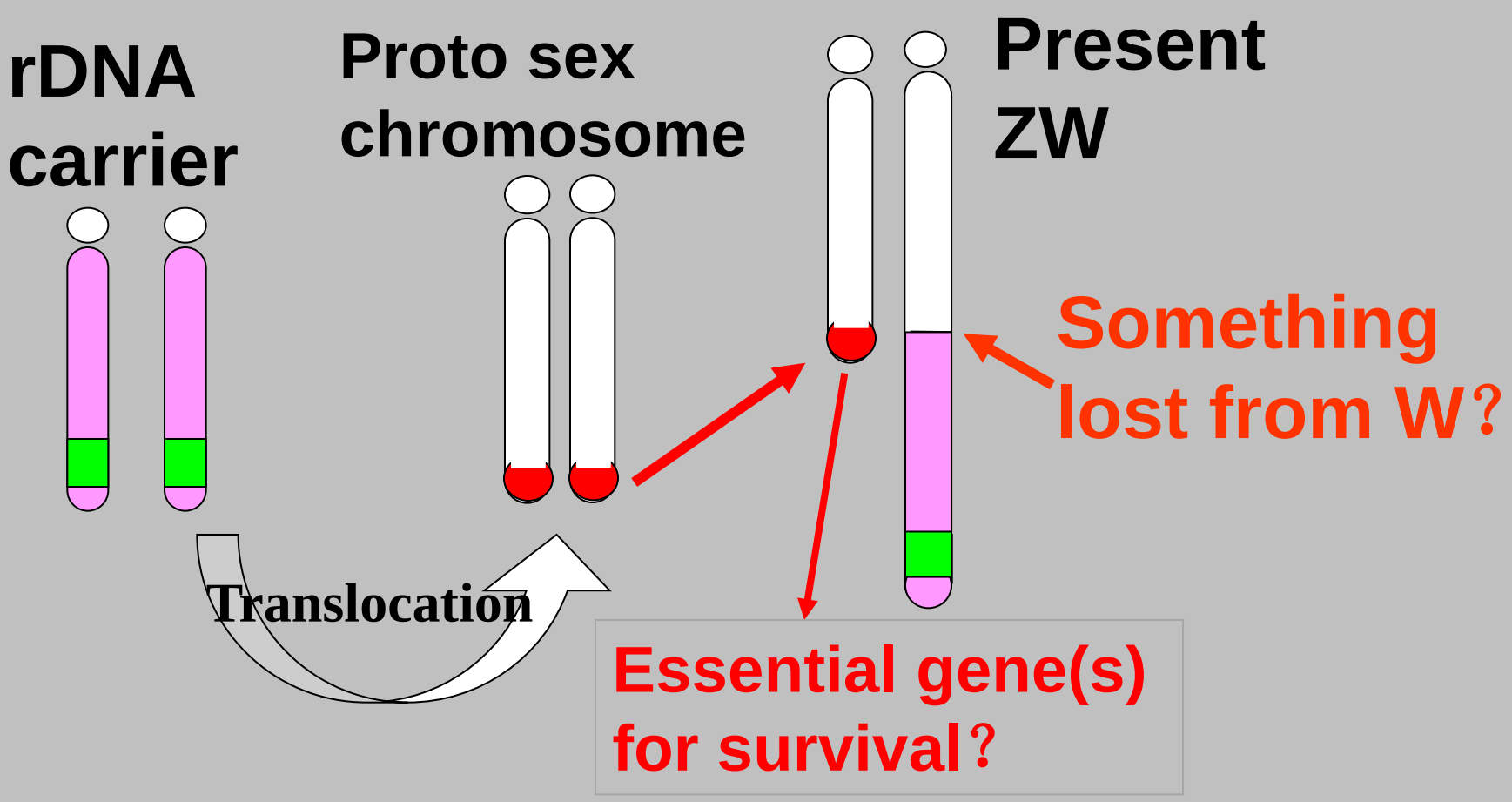
Chromosome (genetic sex)	Gonadal ♀		Gonadal ♂	
	No.	Average Body weight	No.	Average Body weight
ZZ	2	30.1	14	29.9
ZW	12	35.2	6	28.4
ZZZ	11	20.6	4	28.3
ZWW	8	35.8	6	17.2

1. Sex of triploids were twisted,
   Most ZZZ were females, and half ZWW were males
2. Sex reversed ones grew worse than un-reversed ones
3. Growth performance of triploids was not good

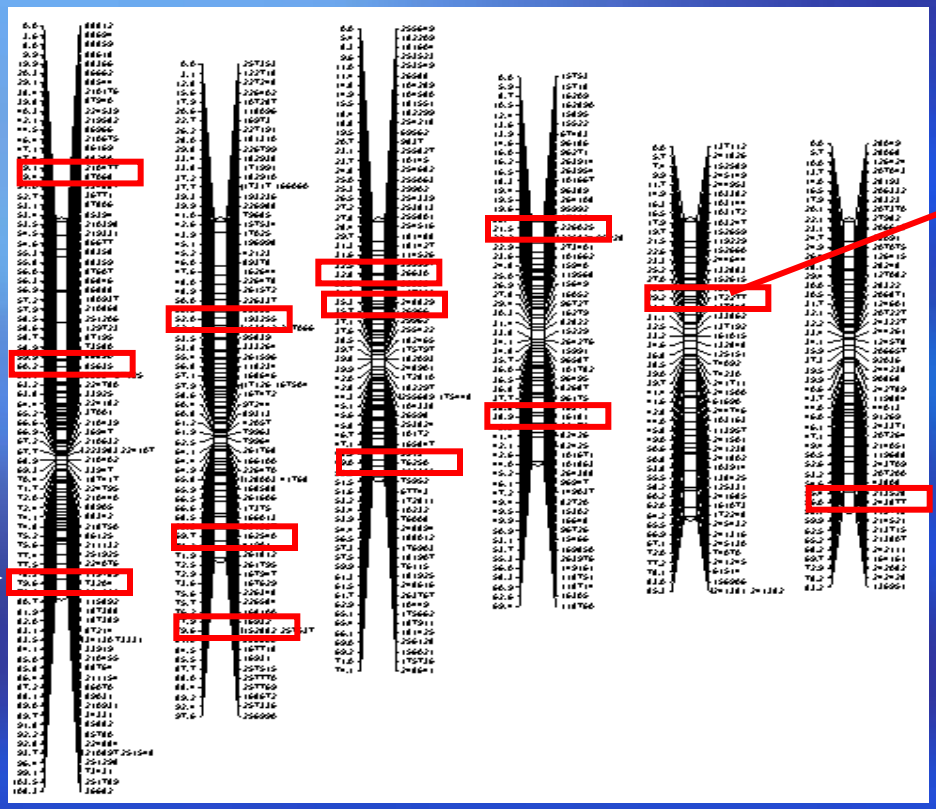
Again, the origin of the W chromosome

Z chromosome is essential for survival

-----Z specific region or genes?



Merge of genetic map and physical map



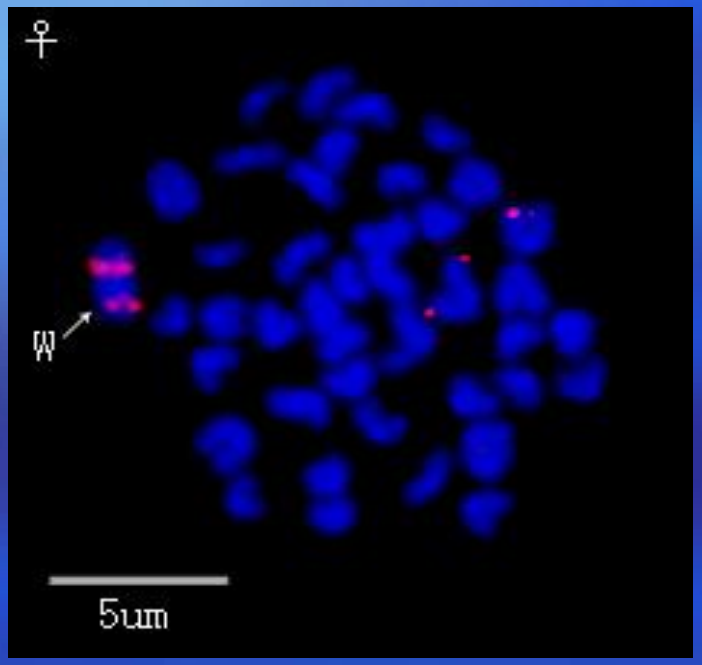
In the genetic map some marekers were derived from Fosmid paired-end sequencing.

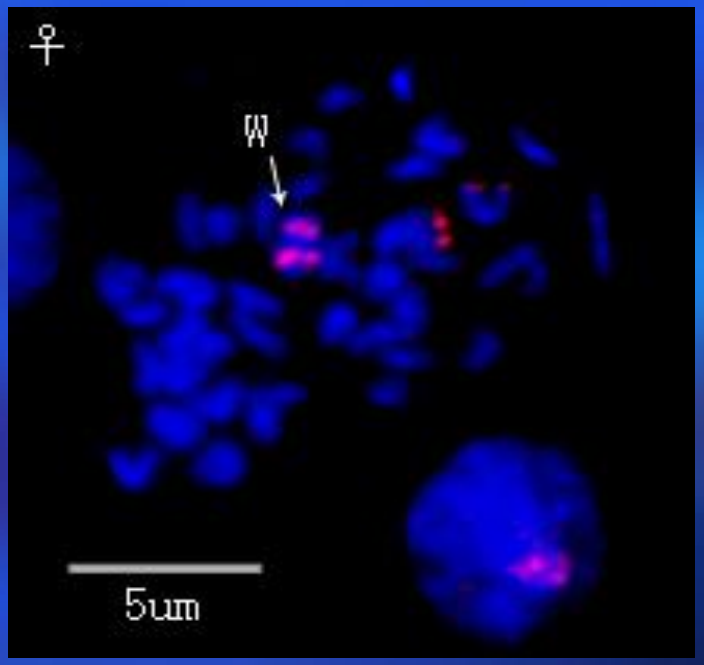
Fosmid clone

ATGGCATCCAGA-----**ATATATATATATATATATATATAT**------GGCATCCAG

In FISH mapping of Fosmid clones

- We found clone B106-62



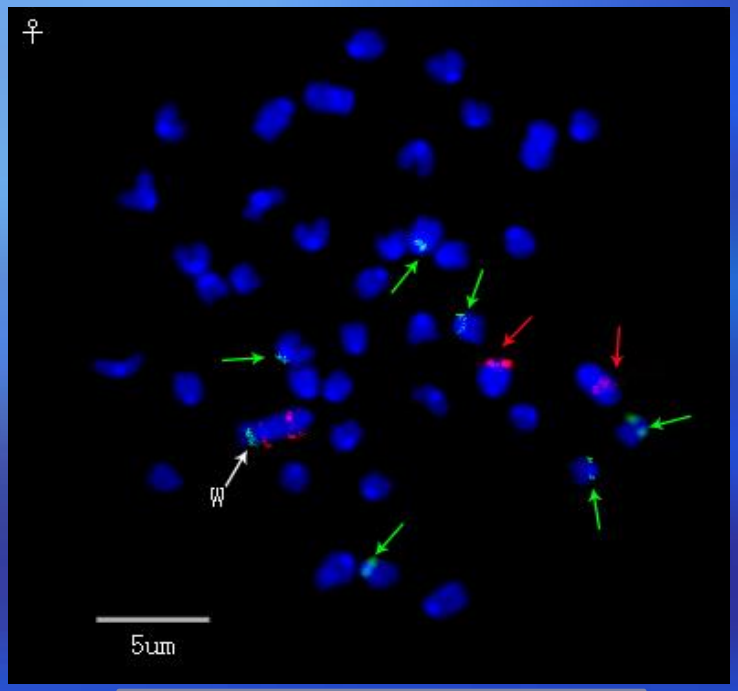


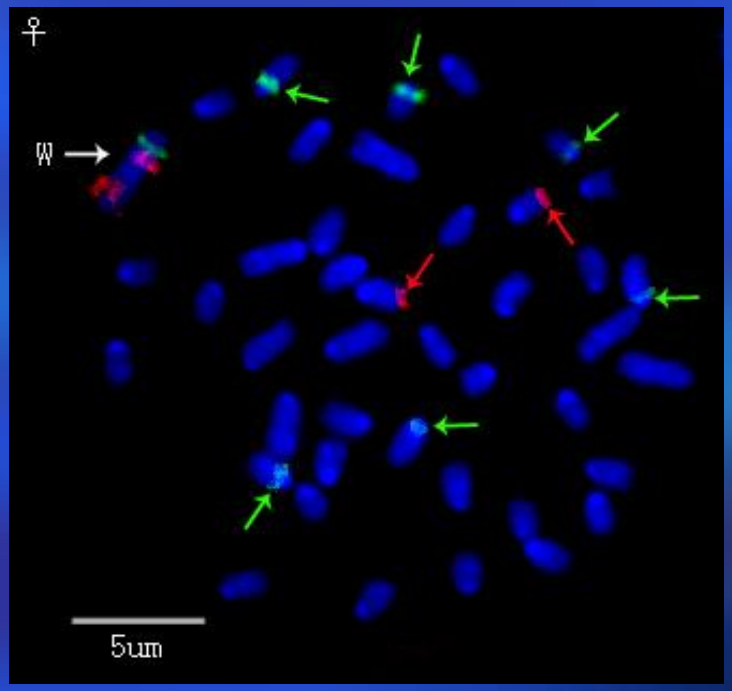
Male: one pair of autosomes

Female: one pair of autosomes

and 2 signals on W chromosome

Two-color FISH——18S + B106-62





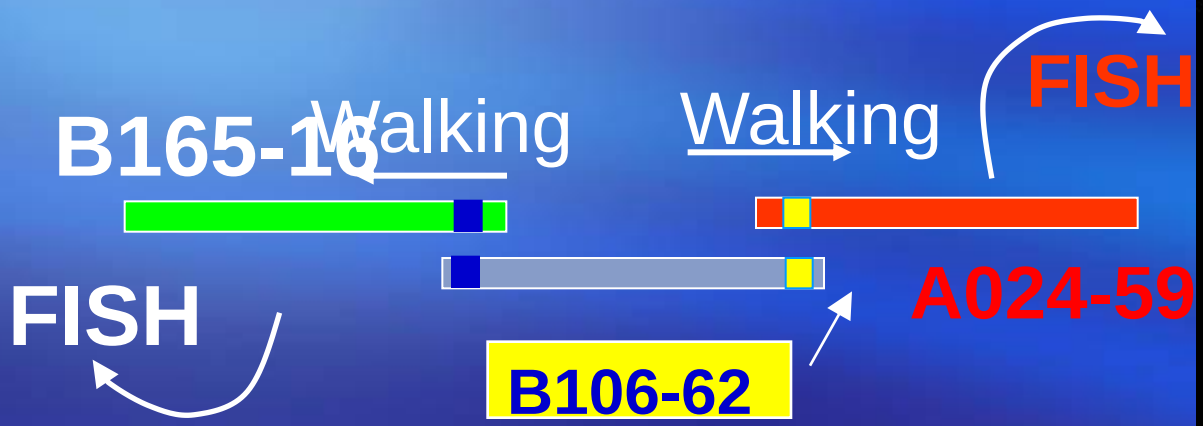
Green： 18S

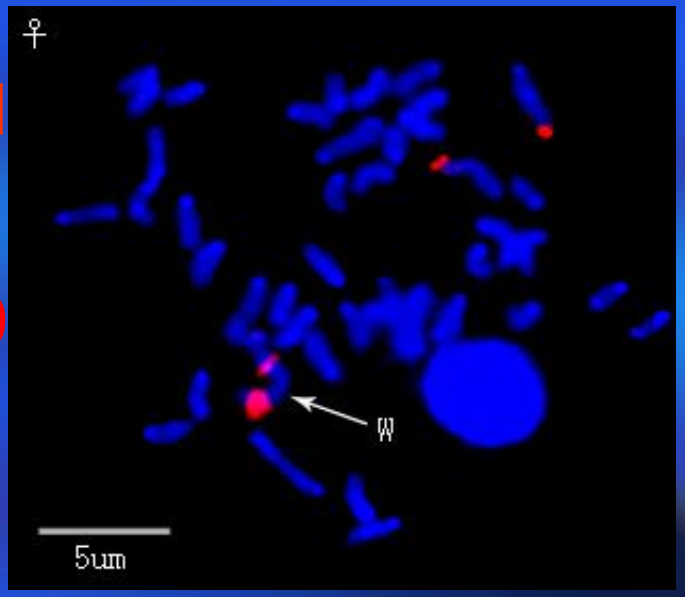
Red： B106-62

Two kinds of signals coexisted on W chromosome.

On autosomes they were separate

Chromosome walking using from B106-62





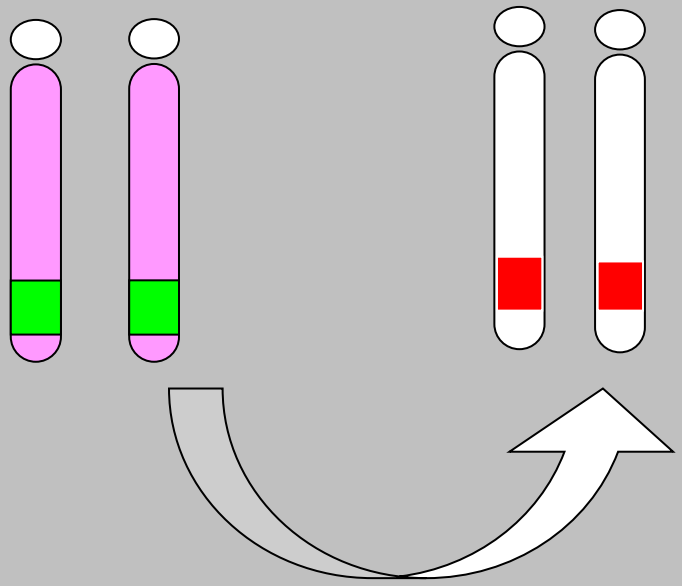
The same FISH signals on W as B106-62

The origin of W chromosome

rDNA Carrier

Proto sex chromosome
B106-62, A024-59,
B165-16 carrier

Present W



translocation

Sex chromosome evolution such as fragment loss and repetitive sequence accumulation

Conclusion

- W chromosome was dissected and W specific library were constructed
- W specific markers identified and molecular sexing methods developed
- WW super female is inviable

  Either by gynogenesis or ZW X ZW crosses
- Triploids were successfully induced, sex of triploids were twisted
- Growth of triploids was not good
- Sex chromosome may be a “recent” evolutionary event in the tongue sole

Acknowledgement

Xubo WANG
Xiaohua SUN
Haiyang YU
Zhigang WANG
Ji QI
Chunmei LI
Liming JIANG

All other members in my lab who have contributed to this work

Thank you !