

## BIODIVERSITY OF FLUKES

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### Summary:

As many others parasites, speciation of flukes depends on the genetic characteristics and on ploidia. Ploidia of flukes can be different in a same species. In Asia, diploid, triploid and hybrid ( $2n/3n$ ) populations are encountered. The comparison of morphological parameters between diploid and triploid flukes showed that they were morphologically different. Nevertheless, a genetic relationship between parthenogenetic organisms would exist regardless of their ploidia. In the *Fasciola* genus, the main consequence of the high level of diversity is the frequent probability of development of resistance to anthelmintics and fast adaptation to climatic changes. In the *Paragonimus* genus, diversity can enhance different forms of pathogenicity, can also be related to the species of intermediate hosts, and to the definitive host. The strain of flukes plays a part in the visceral localization of *P. westermani* adults.

**KEY WORDS** : Digenea, flukes, *Fasciola*, *Paragonimus*, biodiversity, ploidia, speciation.

To assume their survival, parasitic species must reside in their host, even if their life cycle is short. In addition, these species must adapt to a variety of hosts in all stages of evolution (Combes, 1995). The class of Digenea has various specificities which provide many examples of adaptation to host species. Flukes as a whole are comprised of very different species, including at least two hosts, one of which is a mollusk, and requiring ingestion by the definitive host. Flukes have two apparently opposing characteristics in their life cycle. In the mollusk, larval development is clonal because it succeeds the penetration of only one miracidium, which, after asexual polyembryogeny, produces abundant cercariae which can parasitize the definitive host. On the other hand, the definitive host can eat food parasitized by metacercarial cysts produced by various mollusks, and probably from genetically different populations of parasites. The clonal method of larval development in the mollusk raises a question concerning diversity and one can be concerned by the negative impact. Similarly, in *Fasciola*

*hepatica*, cercariae produced by a mollusk are attracted by metacercarial cysts previously fixed on aquatic plants (Vareille-Morel *et al.*, 1998), and we can suppose that the same aquatic plant carries parasitic cysts from the same mollusk, making them genetically quite identical.

## DIFFICULTY OF SPECIATION IN MEDICAL PARASITOLOGY

### PLOIDIA

Parasitology is a biological field in which taxonomy often still uses morphological and Linnean criteria. The limits of the Linnean descriptive methods were reached many decades ago: the dilemma *Entamoeba histolytica/E. dispar* is a demonstrative example.

Similarly, the infra-specific relationships between biogeography of parasites and their biological characteristics (rhythm, pathology, reservoirs) were used to organize the taxonomy of genetically different parasitic populations (*Wuchereria bancrofti*, for instance).

Genetic diversity cannot yet be evaluated by easy and low cost methods. The results of the biological diagnosis of parasitic diseases tend to be less precise, and decrease at the genus level (*E. histolytica/E. dispar*), or even the family (Opisthorchiidae, Ancylostomatidae, Paragonimidae). Protozoa are parasites for which genetic diversity can be evaluated quite easily. Helminths, particularly Digenea, cannot be analyzed easily, because many species have a variable ploidia, which modifies their morphology and DNA size.

A comparative morphometric study, by light microscopy, of eggs and adults from three populations with different sizes of *Fasciola gigantica*, and the morphology of adults, under scanning electron microscopy, showed variations in the sizes of eggs and adults, whereas the surface was similar using electronic morphology (Srimuzipo *et al.*, 2000). The observations on mitotic metaphase chromosomes revealed a diploid type ( $2n = 20$ ) in all three size-races. The speciation of Digenea can no longer be evaluated by simple morpholo-

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gical observation, but requires a preliminary study of ploidy of selected parasitic populations.

#### POLYPLOIDIZATION

Animals are very susceptible to major modifications of their genetic structure. Nevertheless, speciation by polyploidization is more unusual in the animal than in the plant kingdom. Most species are diploid and have two complete sets of chromosomes, one from their mother and the other from their father. However, some individuals from usually diploid species can be polyploid. This polyploidy prevents them from producing fertile descendants with normal individuals of their species. This type of speciation gives quite instantaneous results, because they only occur during one generation.

### PLOIDIA OF FLUKES

The study of karyotypes from *F. hepatica* samples identified triploid azoospermic flukes, which then induced asexual reproduction (Fletcher *et al.*, 2004). This type of reproduction is frequent in Asia among diploid or triploid flukes, suggesting that parthenogenesis is widely present in *Fasciola*. This has important consequences on the population genetics and the evolution of *Fasciola* genus, especially concerning resistance to anthelmintics.

The variability of *Fasciola* sp ploidy, and the relationships with morphology of adult flukes have been well known for many years (Terasaki *et al.*, 2000). In Asia, diploid, triploid and hybrid ( $2n/3n$ ) populations are encountered. Abnormal spermatogenesis and parthenogenesis are very frequent. The comparison of morphological parameters between diploid and triploid flukes showed that they were morphologically different. Nevertheless, a genetic relationship between parthenogenetic organisms would exist regardless of their ploidy. Many studies have been performed on triploid samples of *Fasciola* sp. (Itagaki & Tsutsumi, 1998; Itagaki *et al.*, 1998). The comparison of ITS2 sequences of nuclear rDNA in *F. hepatica* and *F. gigantica* from various countries all over the world demonstrated two types of sequences in triploids: one is quite similar to *F. hepatica*, the other is close to an Indonesian population of *F. gigantica*. Comparison with the triploid Japanese form of *Fasciola* sp. identified it as *F. gigantica*.

### DIVERSITY OF PARASITES IN THE *PARAGONIMUS* GENUS

Ploidy plays a role in the maturation time for *Paragonimus*: for *Paragonimus westermani*, triploid flukes need two months to reach maturity whereas diploids need 2.5 months (Habe *et al.*, 1996).

The possibility of various means of fertilization for *Paragonimus* is well known (Ihm & Ahn, 1979). The study of six Korean strains of *P. westermani* demonstrated the existence of two means of reproduction: inter-fertilization for flukes stemming from great-sized metacercariae, and parthenogenesis for parasites from small-sized metacercariae.

Diversity related to the definitive host and the strain of flukes plays a part in the visceral localization of *P. westermani* adults. In the case of experimental infection, in carnivores (cats or dogs), most adult parasites live in the lungs, whereas in rodents (rat) or other paratenic host mammals (pig), they are localized mainly in muscles and their development is not complete. The Malaysian strains of *P. westermani* have a development and a distribution in cats or dogs quite different from other parasitic strains (Philippines, Japan, Korea, China). In Malaysia, most reservoirs are wild Felidae and *P. westermani* is the only species identified in these populations (Habe *et al.*, 1996).

Diversity can also be related to the species of intermediate hosts. For *P. westermani*, metacercariae size can be variable according to the strains of crayfish in which they develop. Park *et al.* (2001) identified two sizes of metacercariae (300.3 and 362.0  $\mu\text{m}$ ) in two Crustacea strains (*Cambaroides similis*) living in two different Korean sites. Experimental infestation of dogs with one or the other parasitic strain enhanced the development of adult *P. westermani* with two sizes (respectively,  $8.9 \times 5.6$  mm and  $13.3 \times 9.4$  mm), and the production of eggs with different sizes (respectively,  $72.1 \times 46.8$   $\mu\text{m}$  and  $93.5 \times 54.2$   $\mu\text{m}$ ). A species of parasitized mollusks was identified in the biotope where the largest metacercariae lived in crayfish: 0.12 % of 4,218 *Semisulcospira tegulata* snails contained *P. westermani* cercariae. Karyotypes of two *P. westermani* strains revealed the existence of diploid flukes ( $2n = 22$ ) in the strain of small-sized stages and triploids ( $3n = 33$ ) in the other. This triploid strain was then identified as *Paragonimus pulmonalis*, a species which is become a synonym (Hirai *et al.*, 1985).

Diversity can enhance different forms of pathogenicity. For Blair *et al.* (1997), triploid forms of *P. westermani* are more pathogenic for man than diploid forms. The diploid forms mainly induce pleural cavity injury, whereas triploids mainly induce pulmonary cysts (Miyazaki, 1982).

### DIVERSITY OF PARASITES IN THE *FASCIOLA* GENUS

Various mechanisms of resistance to fasciolosis treatment have appeared in many countries, thus increasing research concerning their origin

for many years. The hypothesis of genetic variations inside the *F. hepatica* species justifies the development of recent studies.

Walker *et al.* (2007) examined the genetic diversity of mitochondrial haplotypes of infra-populations of flukes from cattle or sheep and showed that a herbivore could be infected by at least 10 different haplotypes of the parasite in less than one year. The main consequence of this high level of diversity is the frequent probability of development of resistance to anthelmintics and fast adaptation to climatic changes.

The variability of haplotypes of mitochondrial gene sequences (*nad1* and *cox1*) has been demonstrated by Semyenova *et al.* (2006). Samples of flukes from 20 sites in eastern Europe and China had 4.1 % (*nad1*) and 2.3 % (*cox1*) of variable sites and, respectively, 13 and 10 different haplotypes, with a few or no variations between hosts and/or countries. Two lineages were identified with two main haplotypes. One of them was localized in central Asia, China and Australia, and among all the strains of eastern Europe. The origin could be Asia. The other also came from eastern Europe and central Asia.

A more limited study, developed in Turkey, showed the diversity of *F. hepatica* strains by sequencing the mitochondrial gene *nad1* and the ribosomal part ITS1 (Dosay-Akbulut *et al.*, 2005).

Other previous studies (Marcilla *et al.*, 2002; Ramadan & Saber, 2004) were developed to distinguish *F. hepatica* from *F. gigantica*, particularly in countries where both species are sympatric. Intra-specific and inter-specific comparisons were made between parasites from cattle and/or sheep. Results by RAPD-PCR showed a polymorphism within both species and genetic specificities between species.

- In countries where the two species live, inter-specific differences can be enhanced (by PCR-RFLP, for example) to identify the involved species in man as well as in animals. The basis of speciation is a sequence (618 bp) of the 28S rRNA gene which carries a few different nucleotides for each species, without intra-specific variations. Identification of the species *Fasciola* sp. from Japan was established similarly by the study of mitochondrial DNA: the nucleotide sequences from the two selected countries were similar to those of *F. gigantica*, species regarded as dominant in Japan (Hashimoto *et al.*, 1997).

- In countries where the two species, *F. hepatica* et *F. gigantica*, are sympatric, the existence of natural hybrid parasites was checked, particularly after studies by Agatsuma *et al.* (2000). Two regions of mitochondrial DNA (CO1 and ND1) and nuclear DNA (ITS2 and D2 of 28S DNAr) were sequenced from Korean flukes. The CO1 and ND1 regions were monomorphic and similar to those of *F. gigantica*. On the other hand, the ITS2 and D2 regions were found to be polymorphic:

some flukes possessed a *F. gigantica*-type sequence while the others had a *F. hepatica*-type sequence or showed sequences of both types, thus indicating the existence of different alleles at the loci. These results strongly suggest inter-specific cross-hybridization between the two species coexisting in Korea.

## AUTOFERTILIZATION OF MOLLUSKS

### SELECTION OF STRAINS MORE FAVORABLE TO PARASITE LARVAL DEVELOPMENT

Mollusks, intermediate hosts of Digenea, have an ambivalent genital gland, which gives the possibility of autofertilization, particularly when unfavorable environmental conditions (dryness, frost) reduce the probability of interfertilization. It is not so unusual to see a population of snails growing again from only one survivor. Of course, this case limits genetic exchanges between populations. Moreover, larval development of parasites inside mollusks is pathological for their physiology, mainly for the genital gland, resulting in decreased fertilization. This negative consequence is counter balanced by the low percentage of parasitized mollusks (1 %) which demonstrates the existence of an important reservoir of unparasitized, potentially intermediate host snails. A population without any risk of disruption of fertilization mechanisms is therefore maintained.

## COEVOLUTION OF MOLLUSKS AND DIGENEA

Contrary to the evolutionary indifference that the snail might display towards any single Digenean species, the impact of evolutionary or population-genetic changes in the snail must have an enormous impact on each Digenean species that inhabits it (Blair *et al.*, 2001). The evolutionary fate of the snail may not be influenced by any single Digenean species (unless prevalences are atypically high), but that of the Digenean is influenced by the snail. Digeneans must track snails through time but snails need not reciprocate.

Other terms to consider are host extension and its components, host-switching and host-addition (Blair *et al.*, 2001). Host addition may induce parasite speciation through geographical isolation (allopatric speciation) and/or founder effects involving the parasite population. Parasites may thus be maintained in unrelated snail species at different points along a migratory route. To confirm host addition, it will be necessary to show that the parasites occurring in different hosts and loca-

tions belong to the same species or species group, a task for which molecular genetic techniques are well suited.

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## REFERENCES

- AGATSUMA T., ARAKAWA Y., IWAGAMI M., HONZAKO Y., CAHYANINGSIH U., KANG S.Y. & HONG S.J. Molecular evidence of natural hybridization between *Fasciola hepatica* and *Fasciola gigantica*. *Parasitology International*, 2000, 49 (3), 321-318.
- BLAIR D., AGATSUMA T., WATANABE T., OKAMOTO M. & ITO A. Geographical genetic structure within the human lung fluke, *Paragonimus westermani*, detected from DNA sequences. *Parasitology*, 1997, 115, 411-417.
- COMBES C. Interactions durables. Écologie et évolution du parasitisme. Masson, Paris, 1995, 524 p.
- DOSAY-AKBULUT M., TRUDGETT A. & STANHOPE M. Understanding genetic diversity of the liver fluke *Fasciola hepatica*. *Zeitschrift für Naturforschung*, 2005, 60 (9-10), 774-778.
- FLETCHER H.L., HOEY E.M., ORR N., TRUDGETT A., FAIRWEATHER I. & ROBINSON M.W. The occurrence and significance of triploidy in the liver fluke, *Fasciola hepatica*. *Parasitology*, 2004, 128 (Pt 1), 69-72.
- HABE S., LAI K. P.F., AGATSUMA T., OW-YANG C.K. & KAWASHIMA K. Growth of Malaysian *Paragonimus westermani* in mammals and the mode of transmission of the fluke among mammals. *Japanese Journal of Tropical Medicine and Hygiene*, 1996, 24 (4), 225-232.
- HASHIMOTO K., WATANABE T., LIU C.X., INT I., BLAIR D., OHNISHI S. & AGATSUMA T. Mitochondrial DNA and nuclear DNA indicate that the Japanese *Fasciola* species is *F. gigantica*. *Parasitology Research*, 1997, 83 (3), 200-225.
- HIRAI H., SAKAGUCHI Y., HABE S. & IMAI H.T. C-band analysis of six species of lung flukes, *Paragonimus* spp. (Trematoda: Platyhelminthes) from Japan and Korea. *Zeitschrift für Parasitenkunde*, 1985, 71, 617-629.
- IHM G.S. & AHN Y.K. Comparative studies on type determination of *Paragonimus westermani* in Korea. *Yonsei Journal of Medical Science*, 1979, 12, 38-47.
- ITAGAKI T. & TSUTSUMI K. Triploid form of *Fasciola* in Japan: genetic relationships between *Fasciola hepatica* and *Fasciola gigantica* determined by ITS-2 sequence of nuclear rDNA. *International Journal for Parasitology*, 1998, 28 (5), 777-781.
- ITAGAKI T., TSUTSUMI K.I., ITO K. & TSUTSUMI Y. Taxonomic status of the Japanese triploid forms of *Fasciola*: comparison of mitochondrial ND1 and CO1 sequences with *Fasciola hepatica* and *Fasciola gigantica*. *Journal of Parasitology*, 1998, 84 (2), 445-448.
- MARCILLA A., BARGUES M.D. & MAS-COMA S. A PCR-RFLP assay for the distinction between *Fasciola hepatica* and *Fasciola gigantica*. *Molecular and Cellular Probes*, 2002, 16 (5), 327-333.
- MIYAZAKI I. Geographical distribution of *Paragonimus westermani* and *P. pulmonalis* in Asia. *Medical Bulletin of Fukuoka University*, 1982, 9, 11-22.
- PARK G.M., LEE K. J., IM K.I., PARK H. & YONG T.S. Occurrence of diploid type and a new first intermediate host of a human lung fluke, *Paragonimus westermani*, in Korea. *Experimental Parasitology*, 2001, 99, 206-212.
- RAMADAN N.I. & SABER L.M. Detection of genetic variability in nonhuman isolates of *Fasciola hepatica* and *Fasciola gigantica* by the RAPD-PCR technique. *Journal of the Egyptian Society of Parasitology*, 2004, 34 (2), 679-689.
- SEMYENOVA S.K., MOROZOVA E.V., CHRISANFOVA G.G., GOROKHOV V.V., ARKHIPOV I.A., MOSKVIN A.S., MOVSESSYAN S.O. & RYSKOV A.P. Genetic differentiation in eastern European and western Asian populations of the liver fluke, *Fasciola hepatica*, as revealed by mitochondrial nad1 and cox1 genes. *Journal of Parasitology*, 2006, 92 (3), 525-530.
- SRIMUZIPO P., KOMALAMISRA C., CHOCHOTE W., JITPADKI A., VANICHTHANAKORN P., KEHA P., RIYONG D., SUKONTASAN K., KOMALAMISRA N. & TIPPAWANGKOSOI P. Comparative morphology, morphology of egg and adult surface topography under light and scanning electron microscopies, and metaphase karyotype among three size-races of *Fasciola gigantica* in Thailand. *Southeast Asian Journal of Tropical Medicine and Public Health*, 2000, 31 (2) 366-373.
- TERASAKI K., NODA Y., SHIBAHARA T. & ITAGAKI T. Morphological comparisons and hypotheses on the origin of polyploids in parthenogenetic *Fasciola* sp. *Journal of Parasitology*, 2000, 86 (4), 724-729.
- VAREILLE-MOREL C., ABROUS M., DREYFUSS G. & RONDELAUD D. Oviposition behaviour of *Lymnaea truncatula* infected by the trematode *Fasciola hepatica* under experimental conditions. *Journal of Molluscan Studies*, 1998, 64, 387-391.
- WALKER S.M., PRODÖHL P.A., FLETCHER H.L., HANNA R.E., KANTZOURA V., HOEY E.M. & TRUDGETT A. Evidence for multiple mitochondrial lineages of *Fasciola hepatica* (liver fluke) within infrapopulations from cattle and sheep. *Parasitology Research*, 2007, 101 (1), 117-125.