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XVII Workshop of National Reference Laboratories for Parasites
Istituto Superiore di Sanità
15- 16 September 2022

Multilocus genotyping of *Trichinella* using microsatellites: state of the art and development of a SOP

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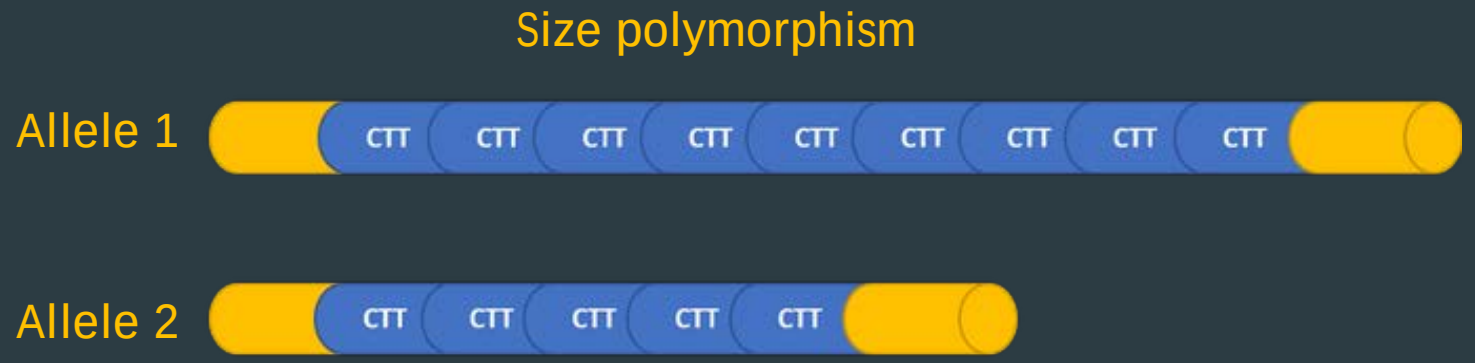
What are microsatellites?

Microsatellites are repetitive DNA elements consisting of tandem repeats of short nucleotide sequences (1 to 6 base pairs in length)

Microsatellites occur at thousands of locations within an organism's genome and have a high mutation rate, caused by expansion/contraction of the repeats, leading to high genetic diversity



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Why to use microsatellites?

Microsatellite alleles are not evenly distributed in the environment and ecological factors in different geographical areas may sustain alternative alleles or different allelic assortments, which could be used to fingerprint parasites of those areas

The analysis of alleles combination across two or more loci (multilocus genotyping) makes possible to acquire information on the genetic structure of isolates circulating in farmed or wild animals

Microsatellites analysis is a useful molecular approach for the traceability (from fork to farm) of *Trichinella*-infected animals, meat and meat products

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Background research

Microsatellites loci have been identified and tested (by Dr. La Rosa and collaborators at the EURLP) on many *T. spiralis* and *T. britovi* isolates of different geographical origin

Collaborative work has been done with NRLs from France, Poland, Spain, Latvia, Estonia and Hungary to carry out investigations on *Trichinella* isolates in specific geographical areas

La Rosa G, Marucci G, Rosenthal BM, Pozio E. Development of a single larva microsatellite analysis to investigate the population structure of Trichinella spiralis. Infect Genet Evol. 12:369–76.2 (2012)

La Rosa G, Calero-Bernal R, Pérez-Martín JE, Tonanzi D, Galati F, Serrano-Aguilera FJ, et al. Rare but evolutionarily consequential outcrossing in a highly inbred zoonotic parasite. Int J Parasitol. 48:543–53.4 (2018)

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T. spiralis outbreaks in Poland

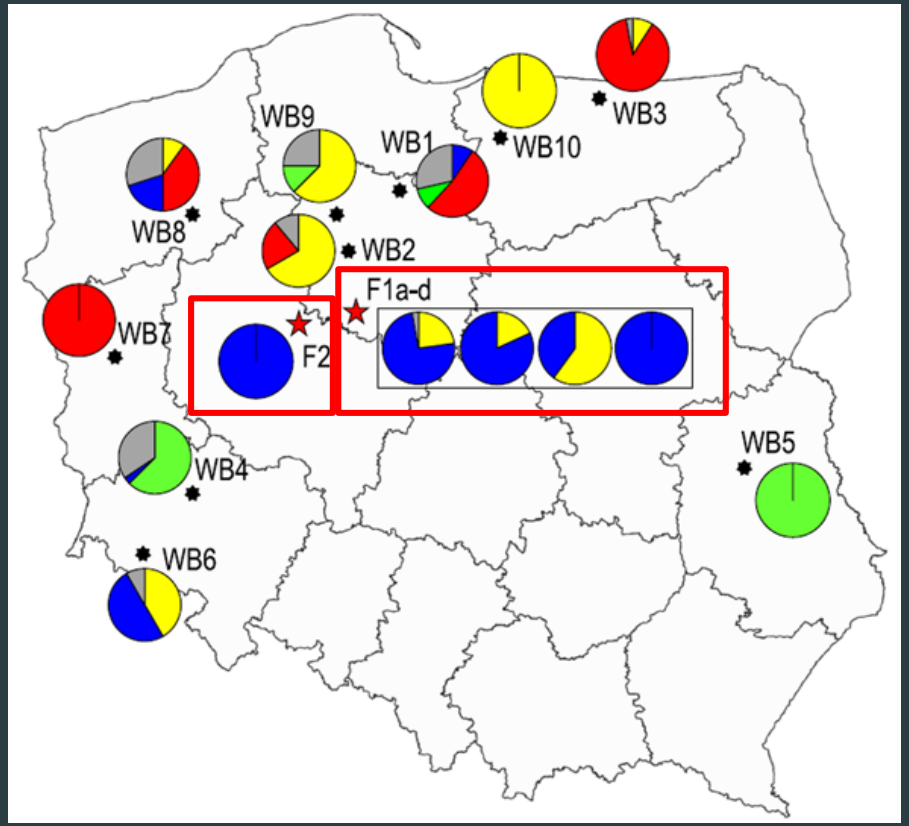
Two outbreaks occurred in backyard pig farms in 2 neighboring provinces of Poland in 2013-2014

9 microsatellite markers were used to examine the genetic structure of *T. spiralis* isolates collected in the two pig farms and from wild boars hunted in several Polish provinces

Genetic uniformity observed in parasite populations of each farm outbreak

High genetic similarity of parasites from the first and second outbreaks suggested an epidemiological link between them

Wild boars harbored more genetically variable larval populations, well distinct from those isolated in domestic pigs



Bilska-Zajac, E., Tonanzi, D., Pozio, E. et al. Genetic evidence substantiates transmission of Trichinella spiralis from one swine farm to another. Parasites Vectors 14, 359 (2021)

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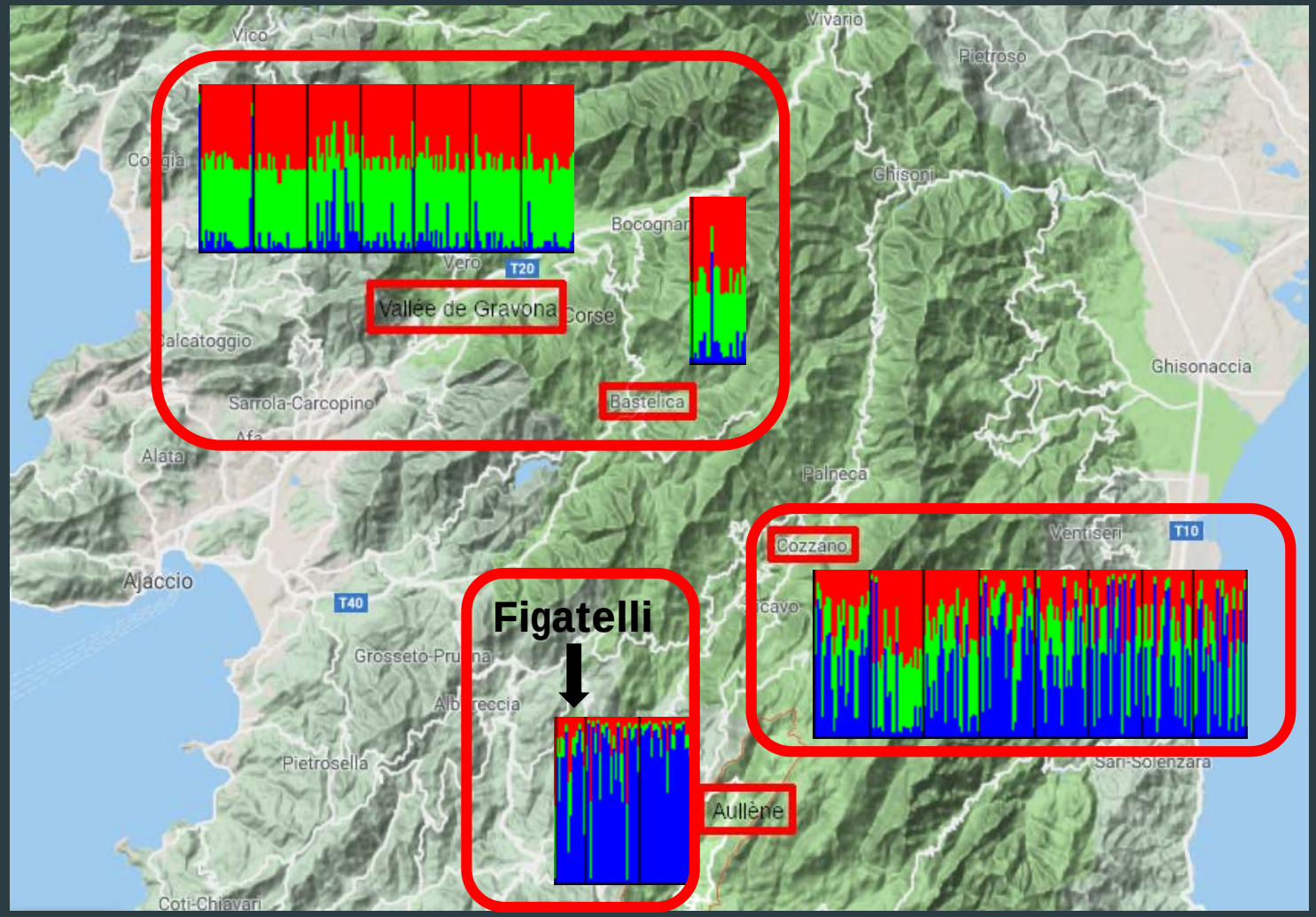
T. britovi populations in Corsica

Human outbreak in Nice (France) in 2015 due to consumption of row sausages made in Corsica (figatelli)

Multilocus genotype of 17 *T. britovi* Corsican isolates was analyzed

Three geographically separated genetic groups were identified on the basis of the type and frequency of the alleles

Larvae isolated from figatelli linked their origin with larvae isolated from an infected sausage collected at the breeder-butcher of Aullène, and with larvae isolated from a domestic pig reared in the same locality



La Rosa, G., Vallée, I., Marucci, G. et al. Multilocus genotype analysis outlines distinct histories for Trichinella britovi in the neighboring Mediterranean islands of Corsica and Sardinia. Parasites Vectors 11, 353 (2018)

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Status of the art

- Several informative microsatellite markers have been identified for the species *T. spiralis* and *T. britovi* and information on microsatellite alleles circulating in wild fauna of some European countries has been acquired

- However, we are still far from a complete distribution map of the genetic variability of the main *Trichinella* species circulating in Europe. Too many geographic areas remain under-represented or simply lack data, and the species *T. nativa*, present in the Northern regions of Europe, has not yet been analyzed

- The experience gained at the EURLP has been used to create a Standard Operating Procedure (SOP). This SOP can be applied when a detailed genetic characterization of *T. spiralis* and *T. britovi* larvae is required (e.g., for outbreak investigations).

- The EURLP can offer training to the NRLs

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A SOP is born

Workflow

- *DNA purification from single larvae (fresh or fixed in ethanol)*
- *Amplification of multiple microsatellites loci*
- *Fragment typing by capillary electrophoresis*
- *Identification of the multilocus genotypes*
- *Analysis of the multilocus genotypes of different isolates to define their relationships*

The procedure in use at the EURLP is based on the use of an automated DNA extraction station and a capillary electrophoresis system. Adjustments to the protocol might be required if different devices are used for DNA extraction or multilocus genotypes identification.

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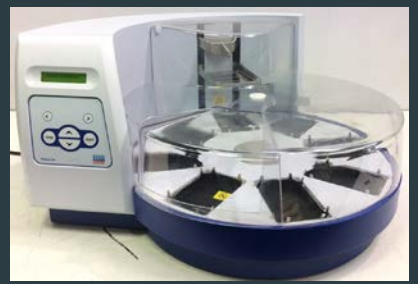
DNA purification

- DNA has to be purified from individual larvae
- To define a reliable genetic structure, 24-36 single larvae for each isolate have to be tested
- A variable percentage of PCR failures can happen depending on the conservation status of the larvae
- DNA is purified by the automated DNA extraction station Biosprint (Qiagen)





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Microsatellites amplification

- An initial multiplex PCR round is mandatory to confirm the *Trichinella* species and to exclude the presence of multiple infections
- Next, PCR with specific primers are performed for each microsatellite locus
- PCR fragments are run on the Qiaxcel capillary electrophoresis sytem (Qiagen) using an high resolution cartridge

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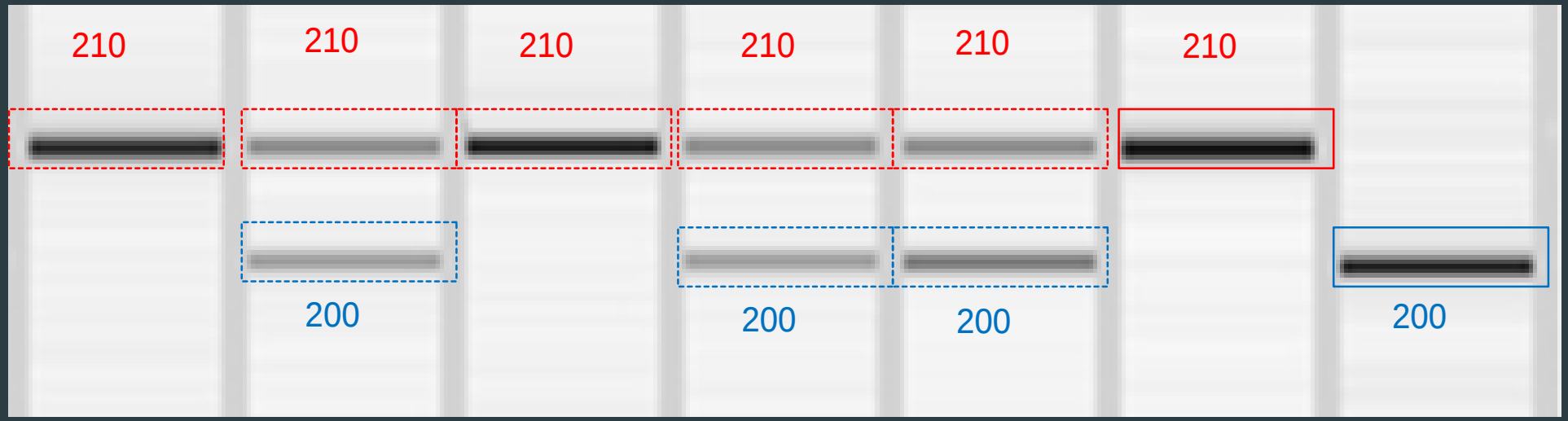
Microsatellites identification

- Homozygotes present in the gel are identified, purified and sequenced
- The sequence length is used to encode the individual alleles (e.g. 200 and 210)
- The genotype of each individual is defined using sequenced alleles as size reference follow the rule: same size, same allele



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Multilocus genotyping

Multilocus genotype (the combination of alleles across two or more loci) of different isolates are compared to define their relationships

	Microsatellite loci					
Isolate	103	128	1007	1122	1380	1010B
Ts1	145	212	174	181	255	250
Ts2	145	212	177	181	267	244
Ts3	145	212	174	181	255	250

Ts1 and Ts3 share the same alleles at the 3 variable microsatellite loci

The genotypic similarity suggest a link between the two isolates

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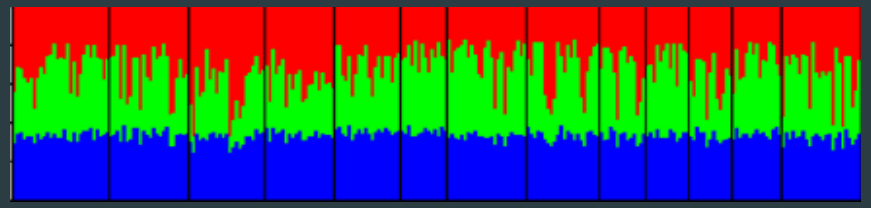
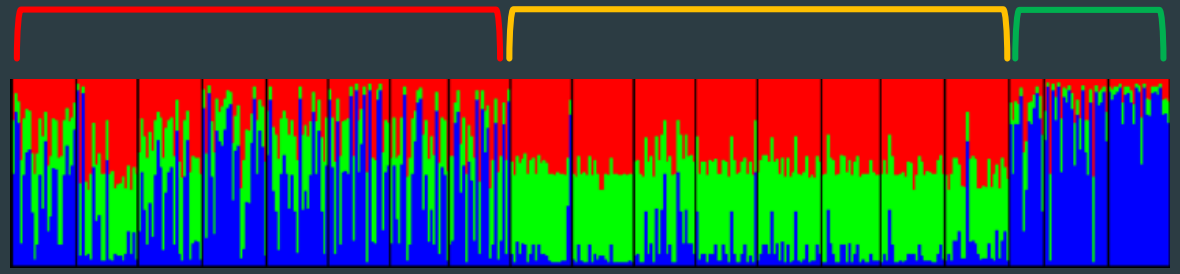
Microsatellite allelic frequencies

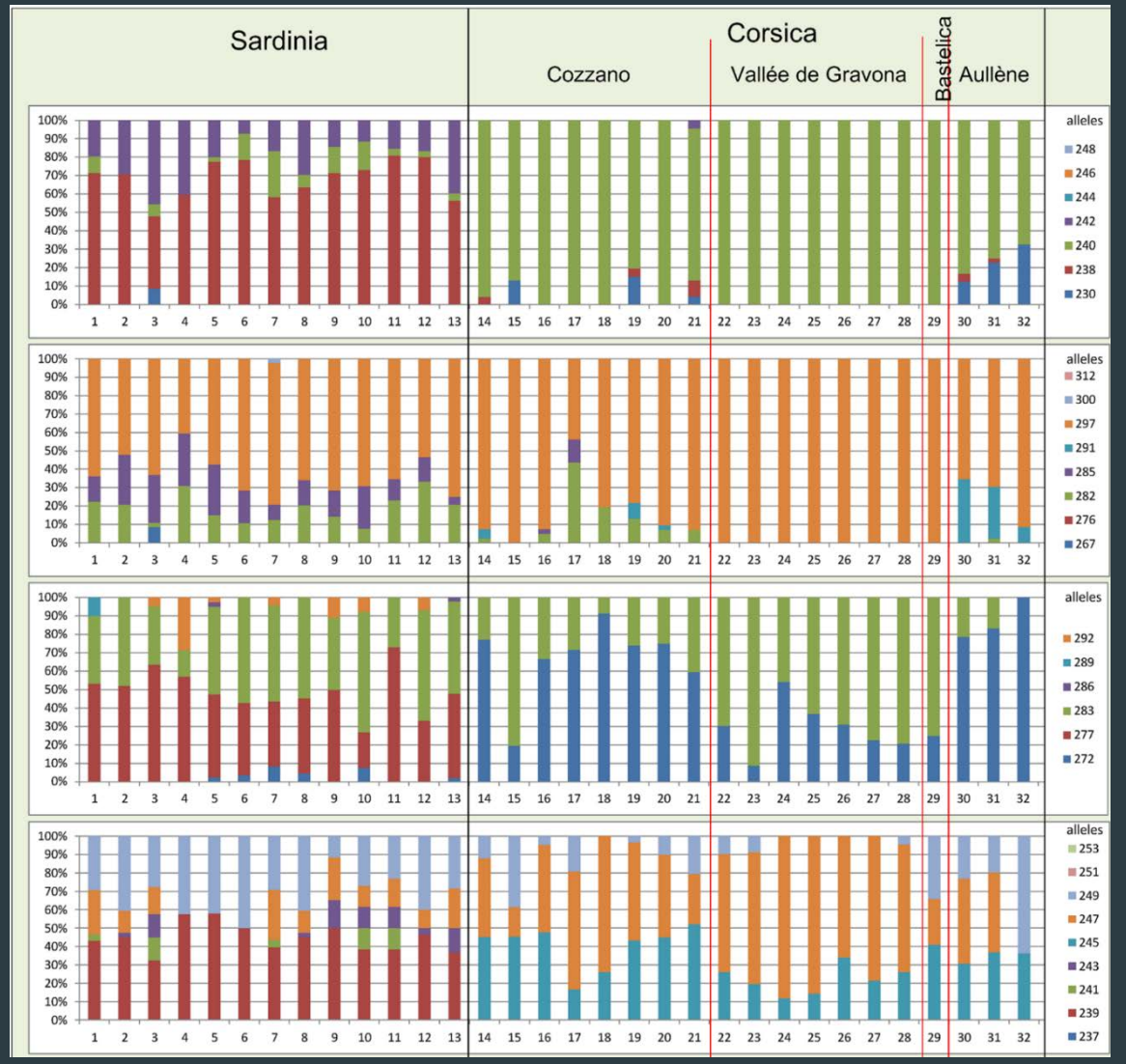
Microsatellite alleles are used as input data in specific softwares to estimate allelic frequencies in specific isolates and to infer the population structure

Sardinian isolates: 1 genetic group



Corsica isolates: 3 different genetic groups





La Rosa, G., Vallée, I., Marucci, G. et al. Multilocus genotype analysis outlines distinct histories for Trichinella britovi in the neighboring Mediterranean islands of Corsica and Sardinia. Parasites Vectors 11, 353 (2018)

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Conclusions

The multi-year study on *Trichinella* microsatellites allowed the production of a SOP that represents a useful tool to support epidemiological investigations during *T. spiralis* or *T. britovi* outbreaks

The information content of the method strictly depends on the previous knowledge on microsatellite alleles that circulate in the specific area and their relative frequencies

Thanks for your attention