Genotyping Toxoplasma: a close collaboration of European laboratories resulted in new guidelines for microsatellite typing and a Next Generation Sequencing-based typing method

WP5

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Population structure of *Toxoplasma gondii*

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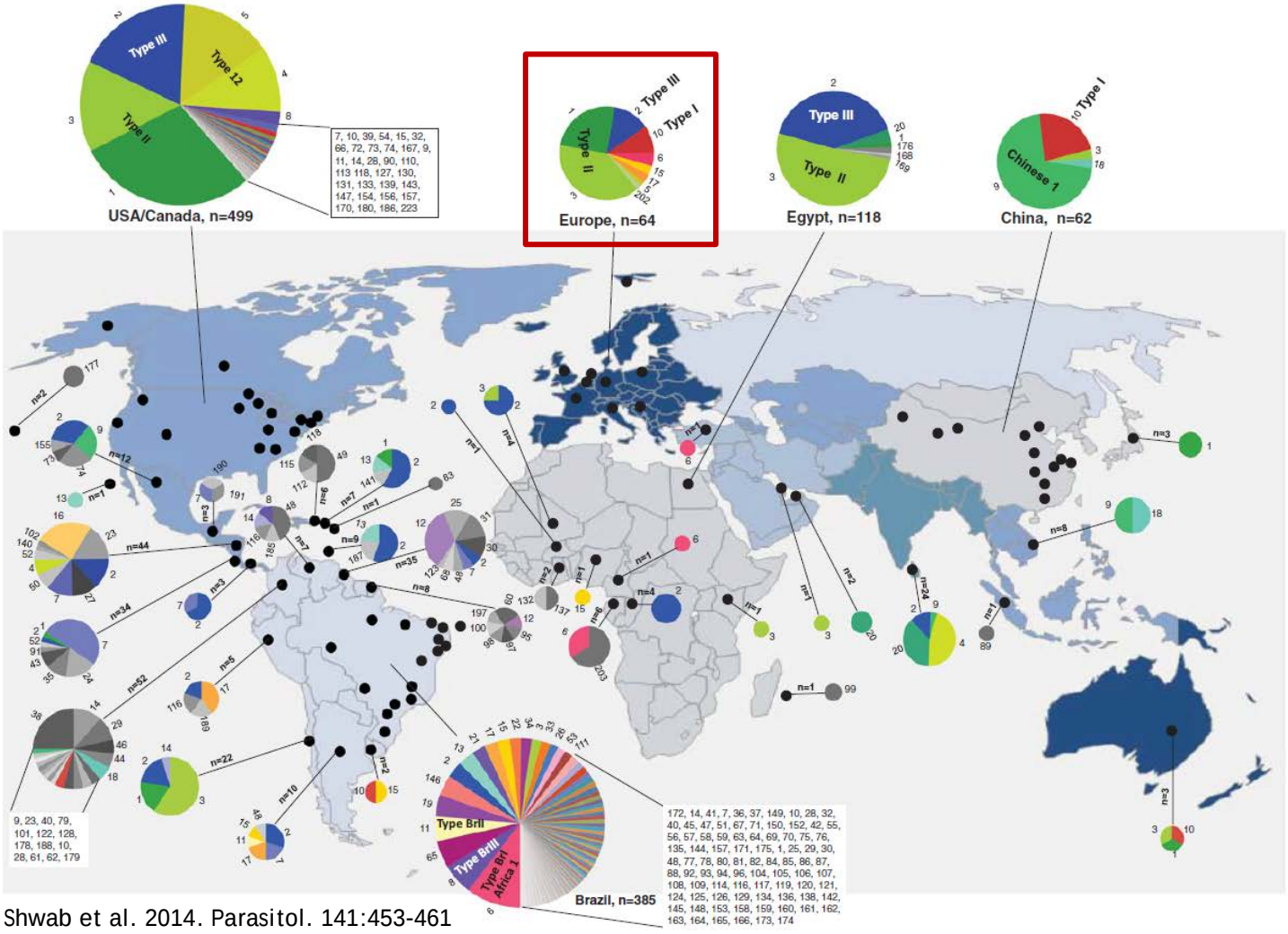
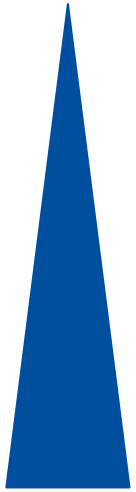
- Many regions of the world dominated by few clonal lineages
- South America: Much more diverse



Shwab et al. 2014. Parasitol. 141:453-461

Tools for population genetics studies in *T. gondii*

Low resolution



High resolution

Multilocus PCR-RFLP markers → SNP based

Multilocus microsatellite typing → short nt repeats

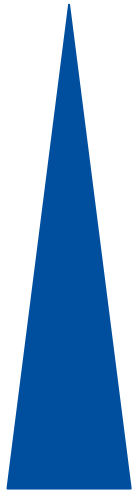
Multilocus sequence typing (MLST) → SNP based

Whole genome sequencing (WGS)

T. gondii genome

- Size: 64 Mb
- Chromosomes: 14

Low costs



High costs

TOXOSOURCES main achievements

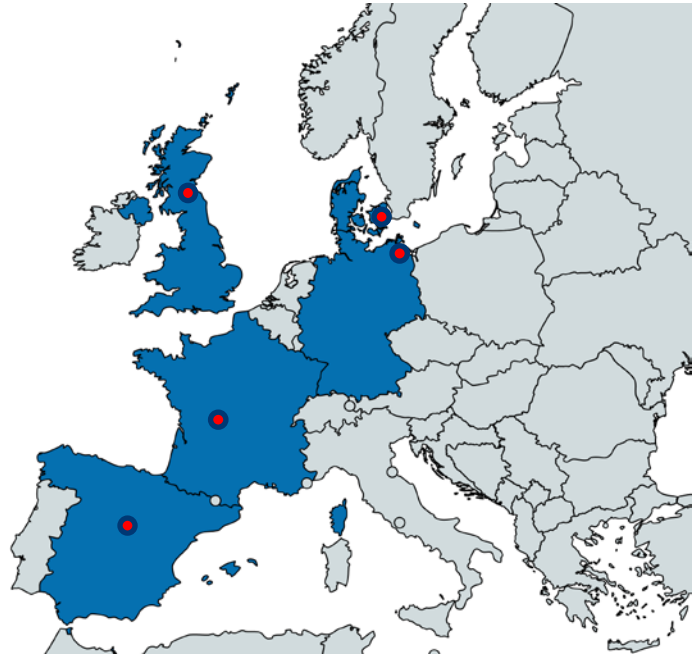
- Ring trial and establishment of **guidelines for microsatellite typing**
  - Adherence to guidelines allows the combination of data sets from different labs

- Large **collection of in-vitro isolates** across Europe
  - WGS for European isolates
  - Toxosources teamed up with the Biological Resources Centre in France and we were able to significantly increase our collection, e.g. by adding non-European samples

- Identification for **targets to fingerprint *T. gondii*** by NGS based typing techniques
  - As an example we established an AmpliSeq typing method
  - Validated this technique using positive clinical samples and isolates DNA

Harmonization of *T. gondii* microsatellite typing

- Participation of five European laboratories in a ring trial
- Three different sample sets:
  - Part 1: Comparison of typing in general, effects of DNA concentration
  - Part 2: Comparison of fingerprinting results (focused on *T. gondii* Type II)
  - Part 3: Typing of non-archetypal genotypes
- Methodological variations were collated using a questionnaire



Countries of origin of participating laboratories

Outcome of ring trial

- Major differences between labs
  - Type of fluorophore to label primers used for microsatellite typing (Atto550 vs TAMRA or NED)

- Minor differences between labs
  - Limited experience
  - Suitability of software
  - DNA concentration
  - Primer supplier (probably caused by differences in chemistry to label primers with fluorophores)

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ORIGINAL ARTICLE

Check for updates

A ring trial to harmonize *Toxoplasma gondii* microsatellite typing: comparative analysis of results and recommendations for optimization

M. Joeres[1] · G. Cardron[1] · K. Passebosc-Faure[2] · N. Plault[3] · M. Fernández-Escobar[4] · C. M. Hamilton[5] · L. O'Brien-Anderson[6] · R. Calero-Bernal[4] · L. Galal[3] · C. Luttermann[7] · P. Maksimov[1] · F. J. Conraths[1] · M. L. Dardé[2,3] · L. M. Ortega-Mora[4] · P. Jokelainen[6] · A. Mercier[2,3] · G. Schares[1]

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Guidelines for microsatellite typing of *Toxoplasma gondii* using fifteen marker regions

Version 1.0; Date: 02.02.2023

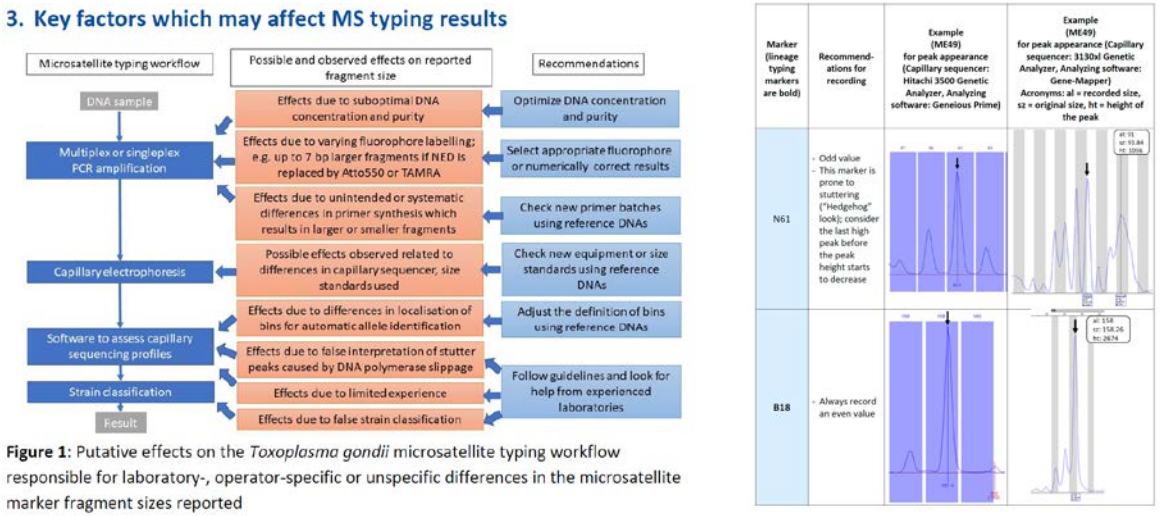


Figure 1: Putative effects on the *Toxoplasma gondii* microsatellite typing workflow responsible for laboratory-, operator-specific or unspecific differences in the microsatellite marker fragment sizes reported

Collection of European *T. gondii* samples

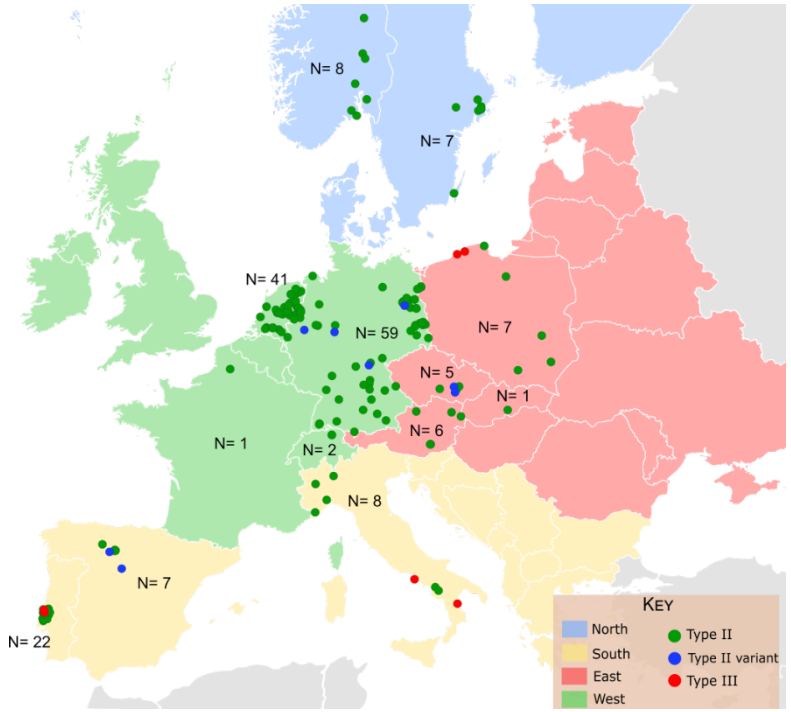
Field sample collection (mainly tissue samples [fixed, non-fixed], oocysts samples)
Many European labs contributed.

- MS typing of 325 European samples (clinical samples and isolates)

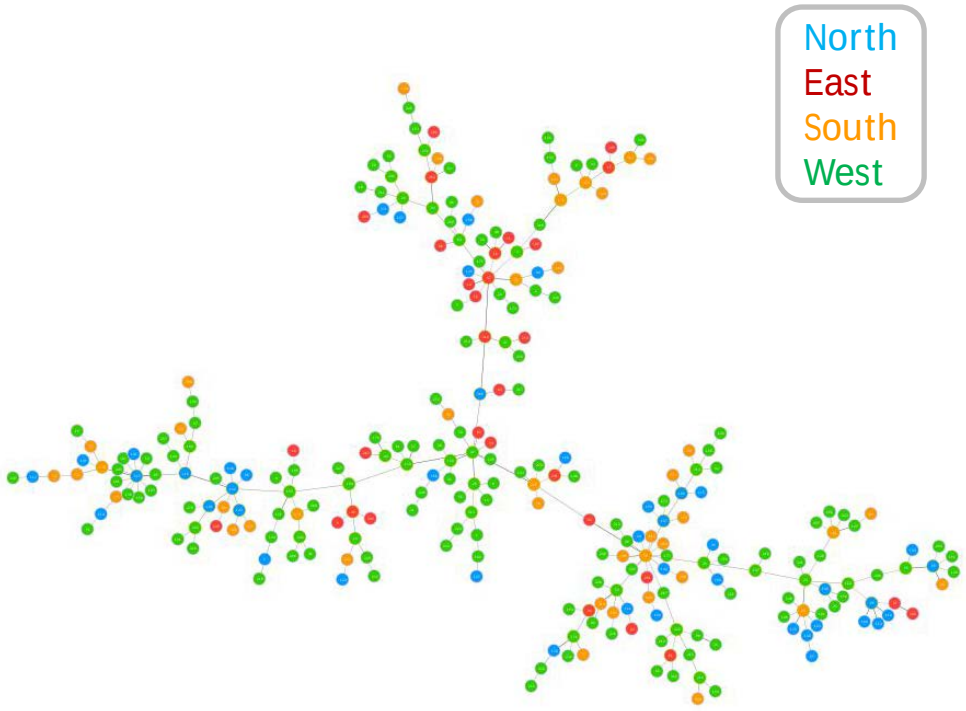
Host category	No. of samples
Human	7
Pets	116
Farm animals	69
Wildlife	116
Zoo animals	17

- All Europe covered

Region	No. of samples
North	49
East	36
South	71
West	169

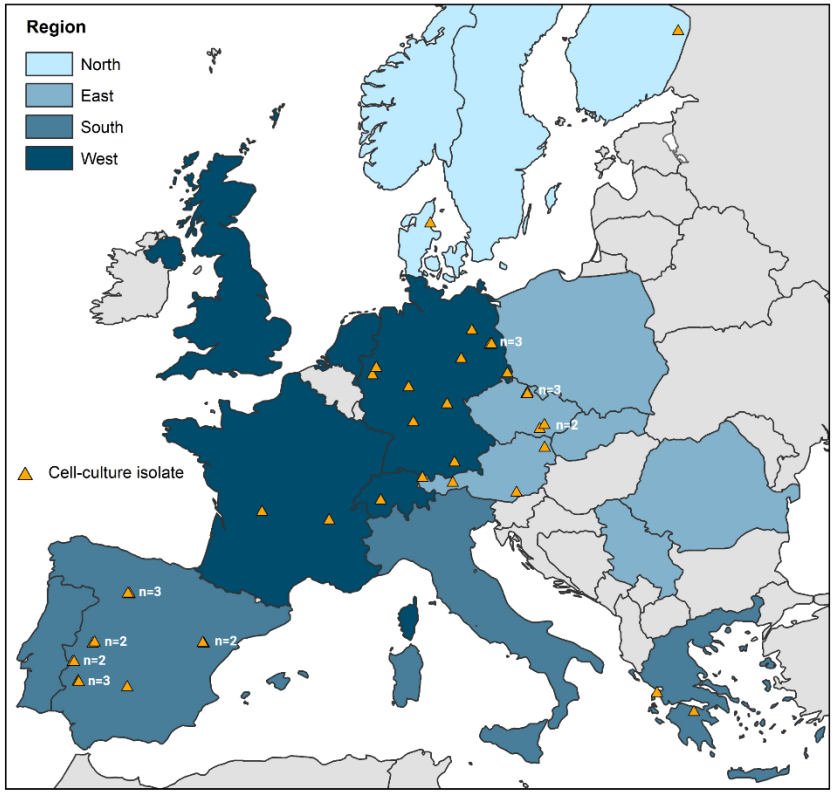


MS typing results of fully typed samples



Phyloviz (goeBURST analysis)

Whole Genome Sequencing (WGS) of European type II reveals a heterogenous SNP distribution



106 cell-cultured isolates from different parts of Europe, focusing type II *T. gondii* whole genome sequenced

SNP numbers in 333 bp frames of the 65 Mbp genome

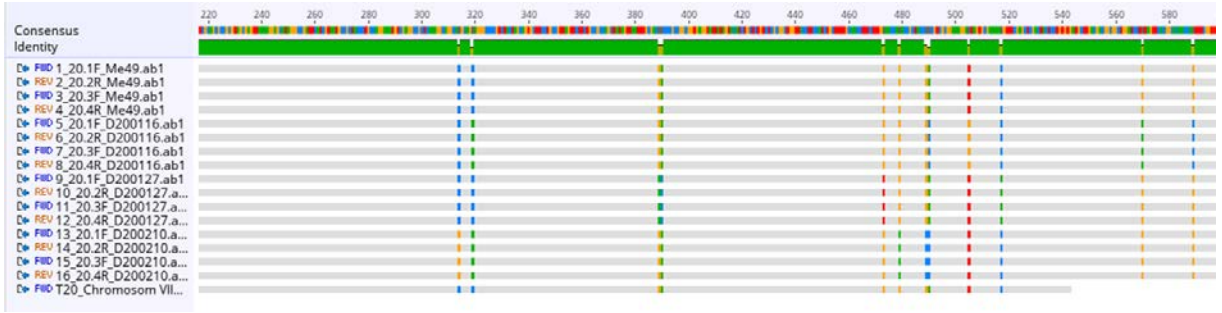
	Number of SNPs in 43 isolates per 333 bp frames						
Chromosome	1-4	5-9	10-14	15-19	20-35	Total	SNPs/10,000 bp
TGME49_chrIa	1158	11	3	0	0	1172	6.30
TGME49_chrIb	1643	20	4	1	0	1668	8.53
TGME49_chrII	1717	38	9	3	0	1767	7.53
TGME49_chrIII	1962	50	19	5	0	2036	8.04
TGME49_chrIV	2145	35	8	1	1	2190	8.15
TGME49_chrIX	4704	77	18	3	1	2794	8.39
TGME49_chrV	2677	68	29	11	9	2543	6.95
TGME49_chrVI	2508	28	5	0	2	3858	8.49
TGME49_chrVIIa	3784	60	6	4	4	3821	7.54
TGME49_chrVIIb	3783	35	3	0	0	5543	7.95
TGME49_chrVIII	5491	41	9	2	0	4803	7.59
TGME49_chrX	5622	132	15	6	1	5776	7.72
TGME49_chrXI	4729	41	5	0	1	4776	7.21
TGME49_chrXII	4804	37	3	1	0	4845	6.83
		673	136	37	19		
		4th priority targets	3rd priority targets	2nd priority targets	1st priority targets		

Confirms previous finding < 10/10 kbp

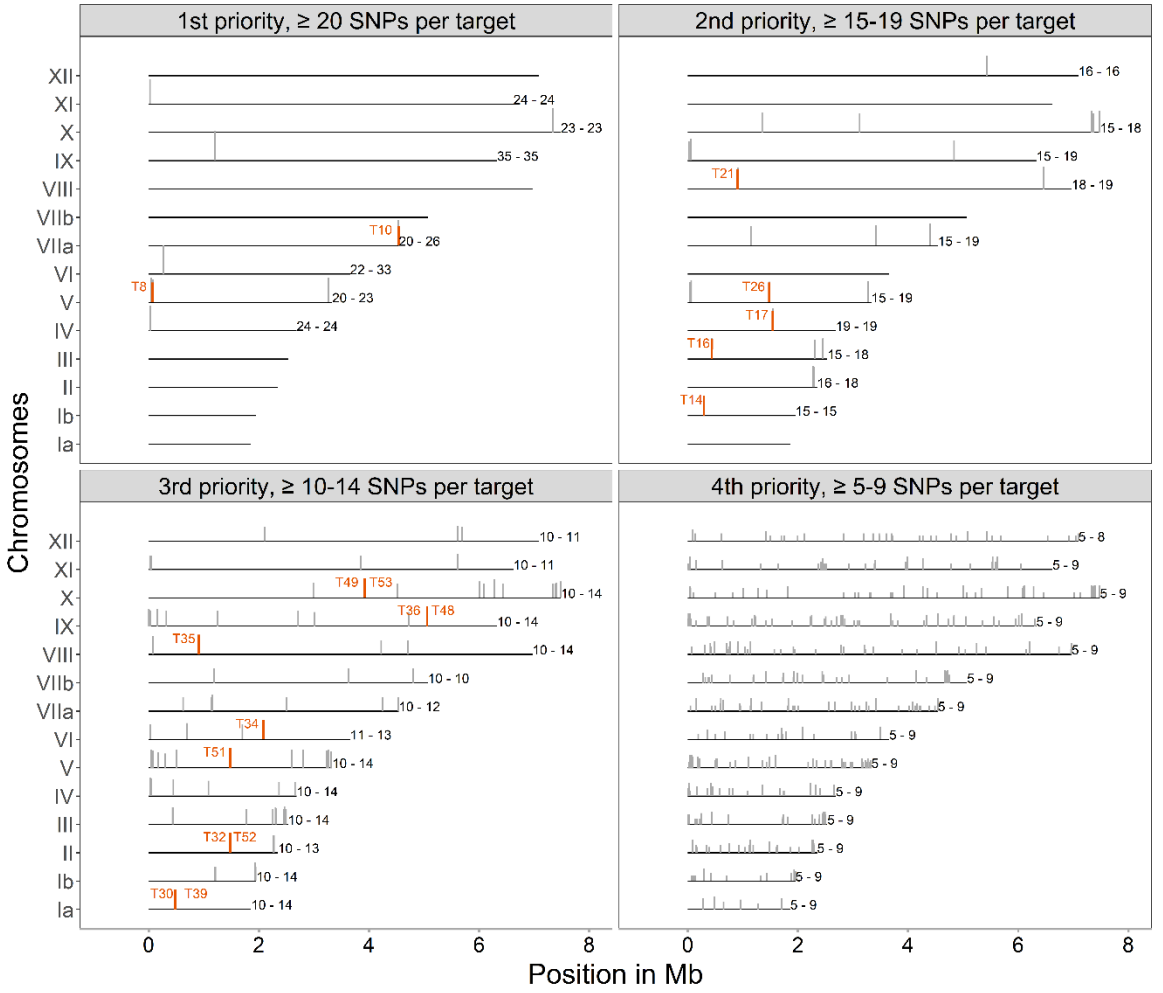
55 potentially suitable regions identfied

SNP rich regions or highly polymorphic regions (HPRs)

Sanger sequencing of 55 potentially suitable regions
-> 17 selected (Chr Ia, Ib, II, III, IV, V, VI, VIIa, VIII, IX, X)

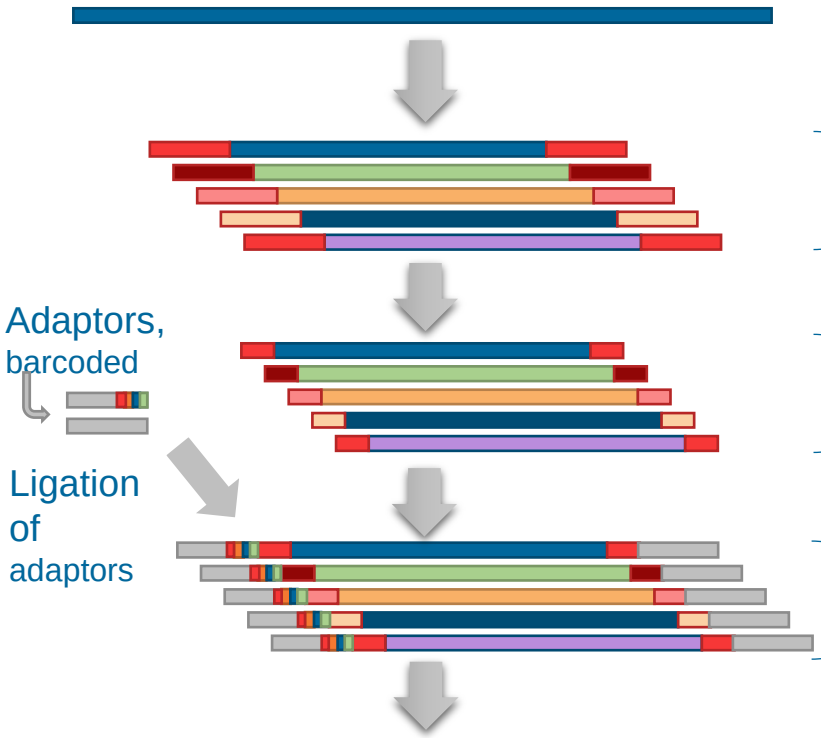


Example of a SNP-rich region

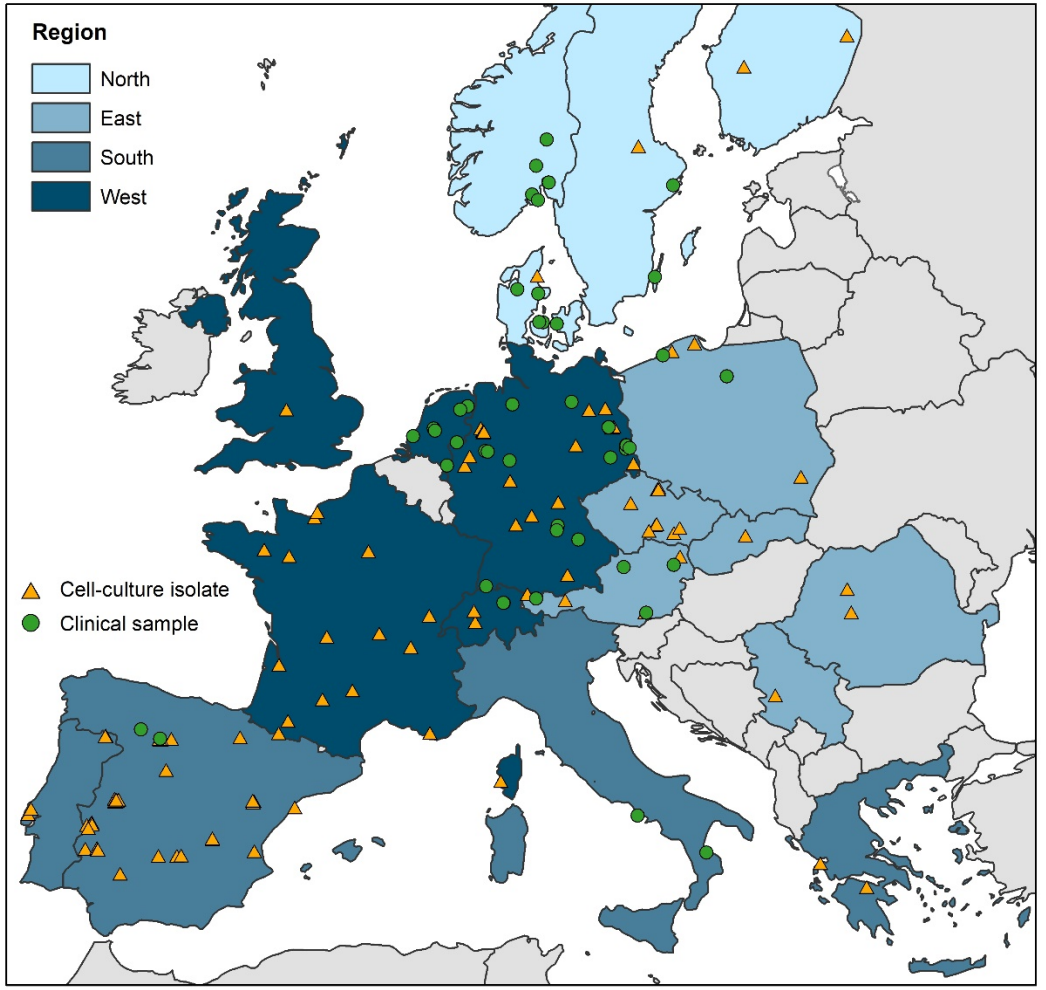


Cell culture and clinical samples used to apply AmpliSeq

AmpliSeq Technique to type *T. gondii*

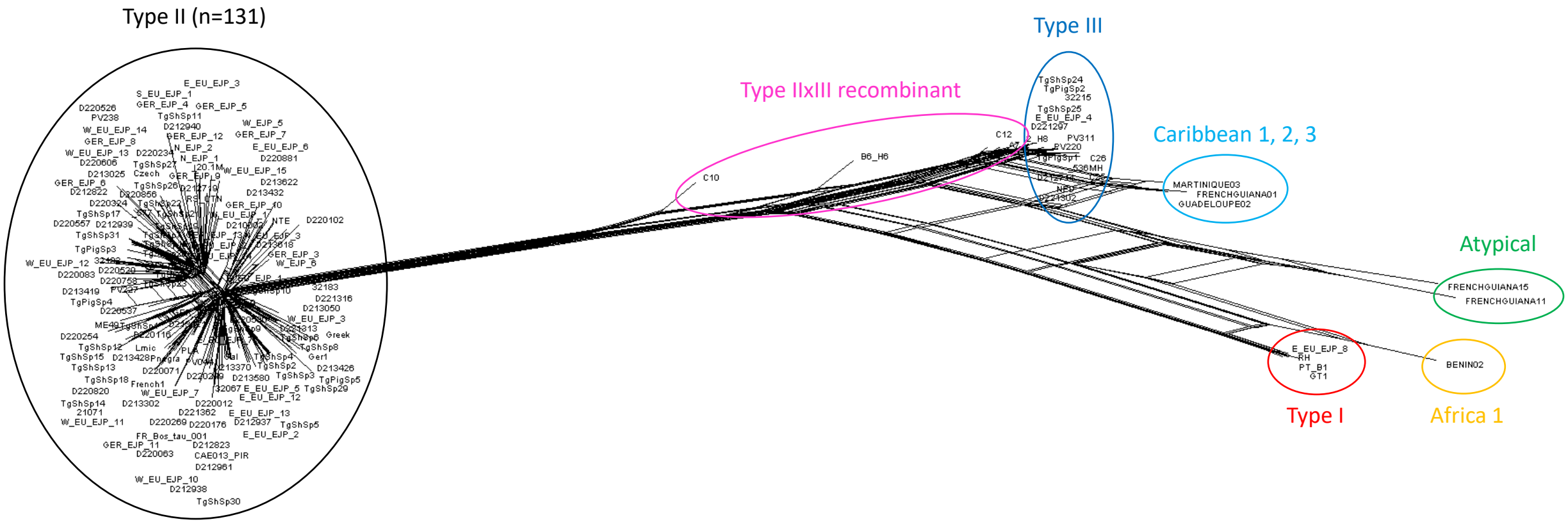


Nest Generation Sequencing (NGS), data analysis



Location of European samples; further exotic samples added (total n=170)

AmpliSeq typing: Clear separation of genotypes within 170 samples



SplitsTree4 (Neighbour-Net)

Fingerprinting of 132 type II samples using 17 regions

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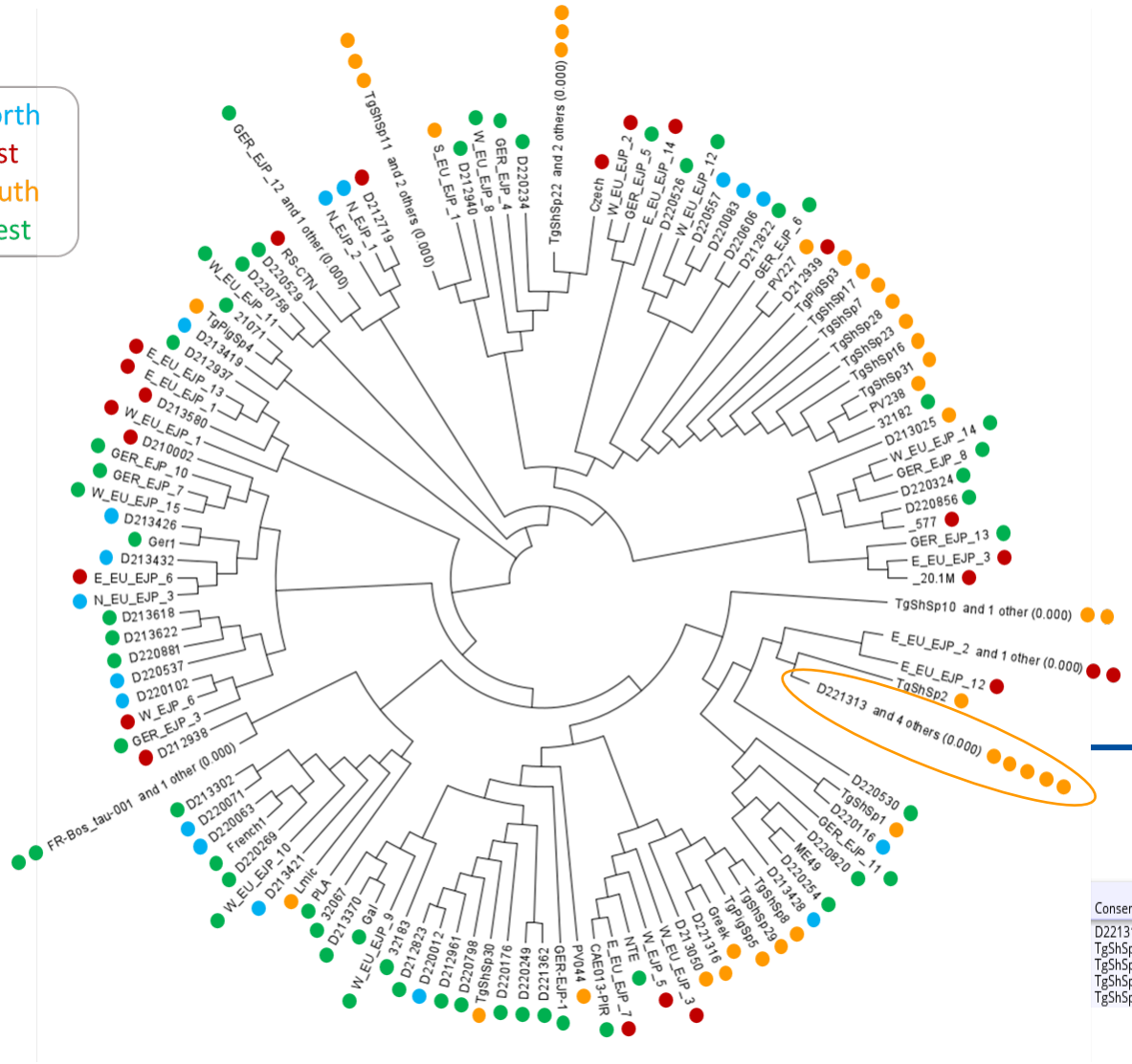
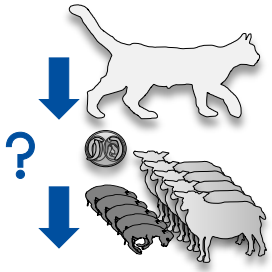
North
East
South
West



- Differentiation within 131 type II samples (including isolates and clinical samples)
  - Ion AmpliSeq: **115** different profiles
  - MS typing: **115** different profiles

Example: Results of one abortion outbreak in a sheep flock



Geneious Prime (Neighbour-Joining Tree)

Analytical sensitivity

Serial dilution of the reference ME49 DNA

- Quantification of the amount of DNA by qPCR
  - Cq values: 23.8, 27.4, 30.3
- Different cycling conditions in multiplex PCR
- Three repetitions of the experiment

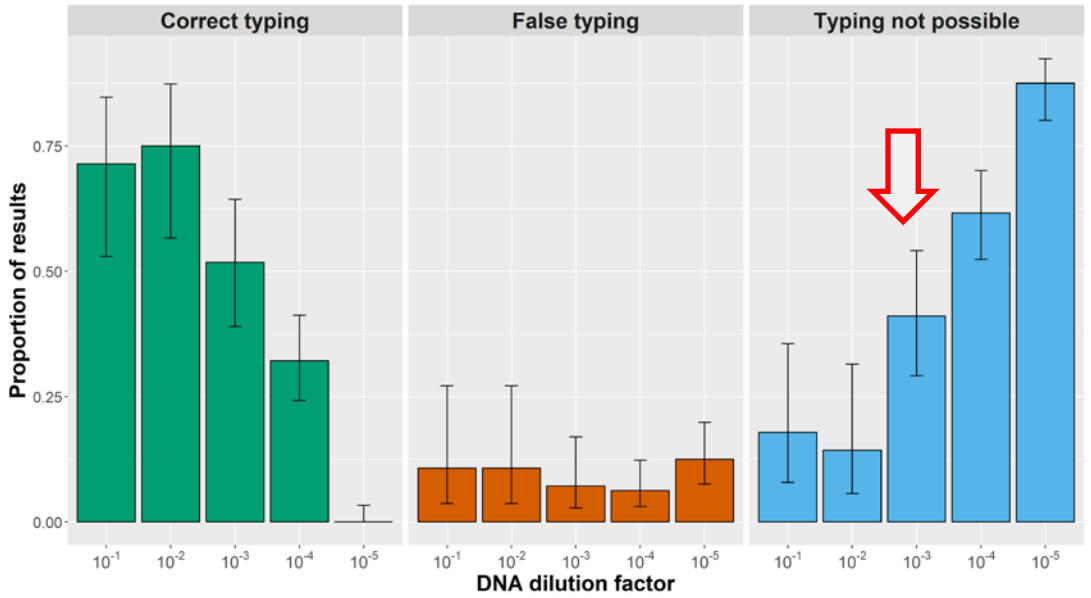
AmpliSeq Typing

	Dilution	PCR Cycles	Covered Regions - Replicate 1	Covered Regions - Replicate 2	Covered Regions - Replicate 3
1 ng/µl [Ct 23.8]	10^{-1}	24	17	17	17
	10^{-1}	26	17	17	17
	10^{-1}	28	17	17	17
0.1 ng/µl [Ct 27.4]	10^{-2}	24	17	17	17
	10^{-2}	26	17	17	17
	10^{-2}	28	17	17	17
0.01 ng/µl [Ct 30.3]	10^{-3}	24	12	10	9
	10^{-3}	26	14	13	9
	10^{-3}	28	16	15	14

Results

- Reduced coverage of targets only in the 3rd dilution
- Better coverage by using more cycles in multiplex PCR
- Sensitivity comparable to the MS typing technique (increased proportion of typing failures in 3rd dilution; 0.01 ng/µl [Ct 30.3 in qPCR])

MS Typing



Conclusion

A large collection of isolates and samples was established

- How to make this collection permanently assessible to other researchers in future?
  (Do we need a European Biological Resources Centre?
  -> Example: Biological Resources Centre for *T. gondii* in Reims, France)

Guidelines for MS Typing were established which allows harmonization of results

An AmpliSeq typing method was established which…

- has a high typing resolution among European *T. gondii* type II samples
- is easy to extend in future and can be automated
- appears promising for tracing infection sources in outbreaks and for detecting recombinant and non-archetypal strains

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Thank you for your attention!

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Why did we use the Ion AmpliSeq platform?

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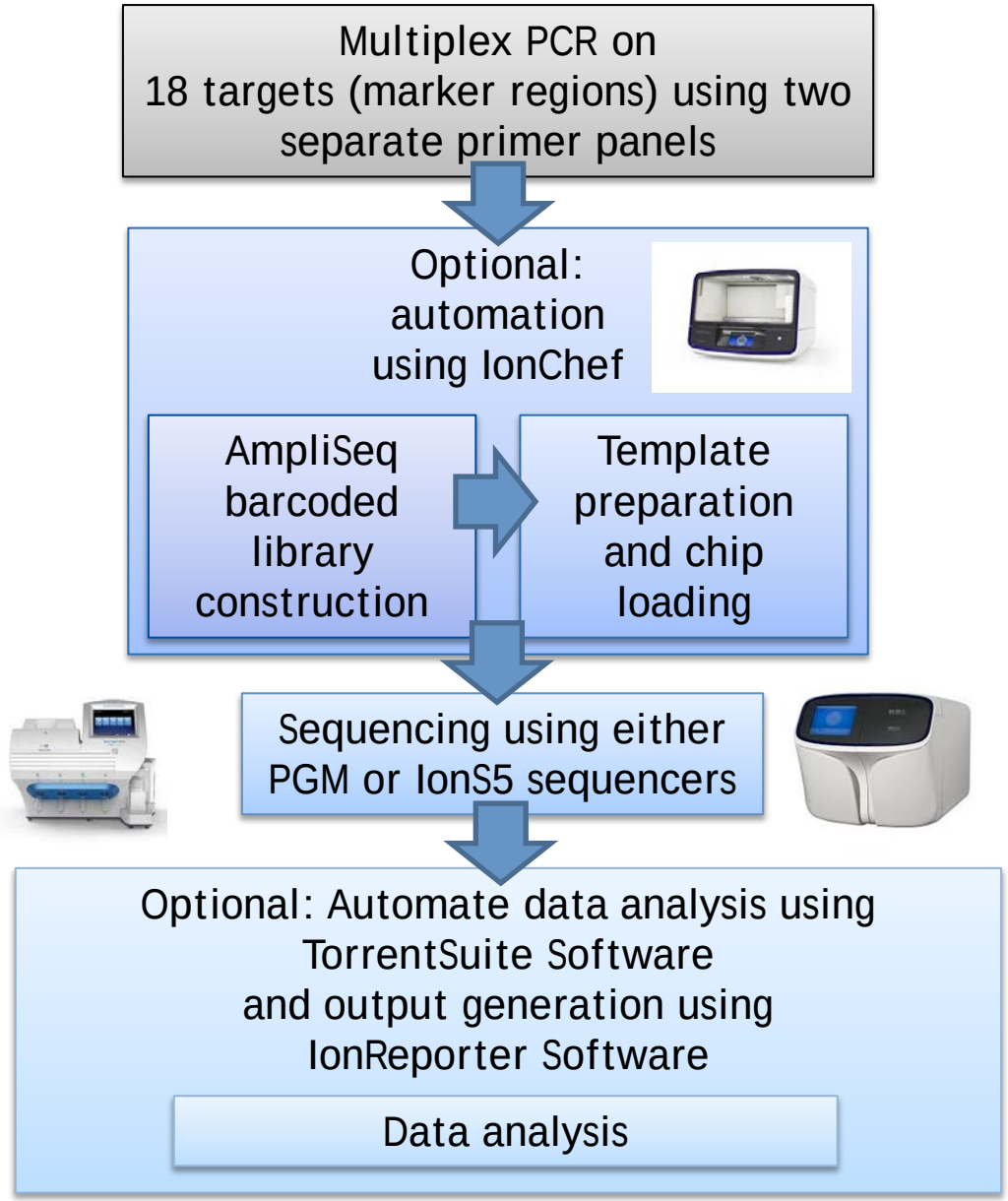
We decided to use Ion AmpliSeq technology because…

- Well established reagents and commercial kits are available
- Typing tool is flexible allowing to add more marker regions (up to 24.000 primer pairs possible!)
- Technology is widely available and used by clinical researchers (E.g. cancer diagnosis, oncology, typing COVID19)



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Task 5.3: European-wide study

- European laboratories contacted to send samples
  → n=1396 samples received, tested for positivity and analyzed by microsatellite typing
- In total n=599 (42.9%) positive
- In total n=215 (15.4%) completely MS typable (i.e. at all 15 MS regions typed)
- For n=174/215 of these precise geographic origin was provided
- Geographic data is missing for some countries
- Predominance of type II