

1 A highly endemic area of *Echinococcus multilocularis* identified
2 through a comparative re-assessment of prevalence in red fox
3 (*Vulpes vulpes*), Alto Adige (Italy: 2019-2020)

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24 ABSTRACT

25 **Background:** Surveillance of *E. multilocularis* at the edge of its range is hindered by fragmented
26 distributional patterns and low prevalence and burden in definitive hosts. Thus, tests with adequate
27 levels of sensitivity are especially important for discriminating between infected and non-infected
28 areas.

29 **Aim:** We reassessed the prevalence of *E. multilocularis* at the southern border of its distribution in
30 Alto Adige (Italy), to improve surveillance in wildlife and provide more accurate estimates of exposure
31 risk.

32 **Methods:** We compared results from the diagnostic test currently implemented for surveillance
33 (based on Coproscopy+Multiplex PCR - CMPCR), against a real-time quantitative PCR (qPCR) for 235
34 fox faeces collected in 2019-2020. The performances of the two tests were estimated using a scraping
35 technique (SFCT) as the gold standard applied to the small intestines of a subsample (n=123) of the
36 same hosts. True prevalence was calculated and sample size required by each faecal test for the
37 detection of the parasite was then estimated.

38 **Results:** True prevalence of *E. multilocularis* in foxes (14.3%) was definitely higher than reported in
39 the last decade (never >5% from 2012 to 2018). The qPCR also had a higher sensitivity (83%)
40 compared to CMPCR (21%). Agreement with the gold standard was far higher for qPCR (0.816) than
41 CMPCR (0.298) as well, determining a smaller sample size required to detect the disease.

42 **Conclusions:** Alto Adige should be considered a highly endemic area. Surveillance at the edges of *E.*
43 *multilocularis* distribution should adopt qPCR diagnostics on definitive hosts on a small geographic
44 scale.

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47 **AUTHOR SUMMARY**

48 *Echinococcus multilocularis* is an intestinal flatworm, whose adult stage in Europe is harboured mainly
49 by the red fox (*Vulpes vulpes*), which spreads parasite's eggs by faeces. This parasite is the agent of a
50 severe and potentially lethal zoonosis, the alveolar echinococcosis, affecting humans after accidental
51 ingestion of parasite's eggs. In the Italian Alpine area, which represents the southernmost border of
52 *E. multilocularis* European range, surveillance is hindered by a fragmented distributional pattern,
53 where presence in foxes has been consistently reported only in few isolated foci in Alto Adige
54 (Bolzano province – Italy) of low prevalence. In order to improve the efficiency of monitoring efforts,
55 we tested the performances of two diagnostic protocols on fox faeces (sedimentation, filtration,
56 counting technique followed by standard PCR, and whole stool real-time PCR) against a benchmark
57 technique on fox intestines (scraping, filtration, counting technique - considered as the gold
58 standard). This allowed not only to determine qPCR as a far more sensitive and sample-efficient
59 diagnostic tool for *E. multilocularis* detection in marginally affected areas, but also to re-assess its
60 prevalence in Alto Adige, which should be considered a highly endemic area. Consequent actions in
61 the field and modifications in the surveillance strategy should be therefore considered.

62

63 **INTRODUCTION**

64 *Echinococcus* spp. (Cestoda, Cyclophyllidea, Taeniidae) are small intestinal tapeworms causing
65 zoonoses of public health importance worldwide. In the European Union (EU), it is mandatory to
66 report the detection of these pathogens to national authorities, and their surveillance, prevention
67 and control are closely regulated for pets, livestock and wildlife.

68 Collection of relevant metadata is also highly recommended by both the Directive EU 2003/99/EC
69 (monitoring of zoonoses and zoonotic agents) and the Regulation EU 2016/429 ('Animal Health Law').

70 Among these, *Echinococcus multilocularis* has a complex life-history. The sylvatic cycle depends on a
71 predator-prey system, where the adult (strobilar) stage of the parasite is carried by wild and domestic
72 canids (definitive hosts: DHs), which in turn become infected by ingesting small rodents (intermediate
73 hosts: IHs) carrying the larval stage (metacestode) of the pathogen [1]. *E. multilocularis* is the agent
74 of a severe zoonosis, alveolar echinococcosis [2], which affects more than 18,000 new patients/year
75 worldwide [3] and more than 150–200/year in the endemic area of central-eastern Europe.

76 In 2019, among the 751 human echinococcosis cases reported in the EU, 147 (26.5%) were
77 attributable to *E. multilocularis* [4], with an incidence which has been increasing notably in recent
78 decades [5]. In humans, who act as dead-end IHs, transmission is predominantly food-borne, and
79 infection occurs when eggs (oncospheres) are ingested with water, wild berries and mushrooms or
80 garden vegetables contaminated with DH faeces [6]. Oncospheres hatch in the gut, penetrate the
81 intestinal wall and by the lympho-hematogenous route reach the liver, where they develop into
82 metacestodes. These multiply asexually, infiltrate the liver and can spread to other organs through a
83 metastasis-like process [7]. Alveolar echinococcosis develops very slowly in humans, taking from a
84 few months [5] to 15 years (<https://www.who.int/news-room/fact-sheets/detail/echinococcosis>) to
85 become clinically evident, although immune-suppressed patients show faster proliferation with
86 earlier detection [8]. If untreated, prognosis is poor, and even the treatment itself is burdensome, as
87 it includes a combination of surgery and long-term anti-parasitic therapy [9].

88 In the EU, the DH is predominantly represented by the red fox (*Vulpes vulpes*). From the historically
89 endemic areas of Switzerland, southeast France and southern Germany, *E. multilocularis* has been
90 expanding north, to northern France and Scandinavia, as well as east to the Balkans [4; 10-12].
91 Moreover, in the last two decades this cestode has been found sporadically in southern France [4;
92 13], as well as northern Italy in both the eastern [14] and western Alps [15], which represents at the
93 moment the southernmost border of the parasite's distribution in the EU. Although high spatial

94 heterogeneity has been noted, in the endemic areas of central and northern Europe the host-
95 parasite-environment pattern seems well established and is expected to be more predictable,
96 whereas the same is not typical of the edges of the distribution [16]. In these areas, long-term
97 surveillance is crucial to assess possible trends in prevalence and spread, as well as the exposure risk
98 for humans. To this aim, sensitivity of the available diagnostic tests is of paramount importance, since
99 worm burden levels are low and the presence of *E. multilocularis* infections in both DHs and IHs may
100 be very hard to detect.

101 In the Province of Bolzano (Alto Adige, northeastern Alps, Italy), *E. multilocularis* in red foxes was first
102 detected in early 2000 at a prevalence of about 13%, estimated using a nested PCR on DNA extracted
103 from fox faeces [14]. In the following years, in order to increase the surveillance area and sample,
104 another test (Coproscopy+Multiplex PCR [CMPCR] on parasite's eggs) was used to analyse 2872 faecal
105 samples across northeastern Italy. While optimizing the cost/benefit ratio [17], this test was known
106 to have a good specificity (93.4%) but a low sensitivity (54.8%), relying on a preliminary screening by
107 flotation of cestodes' eggs, so that their burden could drop under the detection threshold [18].
108 CMPCR was used to confirm the persistence of the Alto Adige focus from 2012 to 2018, with an
109 increasing trend in prevalence in later years, although never higher than 5% annually [17].
110 Considering the persistence and severity of this zoonosis, the current work aimed to enhance the
111 sensitivity of *E. multilocularis* surveillance in red foxes, re-assess its prevalence in Alto Adige, and
112 provide guidelines for a more effective surveillance strategy at the southern edge of its distribution.

113

114 **Methods**

115 In 2019 and 2020, 235 red foxes that were legally culled or found as carcasses were collected across
116 Alto Adige by Provincial wildlife technicians and transported to the Bolzano Laboratory of Istituto
117 Zooprofilattico Sperimentale delle Venezie (IZSve). To achieve an even sampling regime across the

118 territory, one to four animals were collected from each hunting area (Fig 1). At necropsy, a faecal
119 sample was taken from the rectum by a sterile glove. Moreover, when it was intact and not damaged
120 (e.g. by the gun shot), the small intestine was tied off at both ends, removed and stored. Both faecal
121 samples and small intestines were frozen at -80°C for at least 72h to inactivate *Echinococcus* eggs.
122 Each faecal sample was then divided into two equal parts and tested for the presence of *E.*
123 *multilocularis* by two methods:

- 124 1. CMPCR, following Citterio et al. 2021 [17] (at the Parasitology laboratory of IZSVE): briefly, 2
125 g of faecal matter was examined for *Taeniidae* eggs by flotation, filtration and sieving.
126 Multiplex PCR amplification and Sanger sequencing were then performed on harvested eggs.
- 127 2. qPCR following Knapp et al. 2016 [19], with minor modifications (at the Animal Genetics
128 laboratories and Sequencing Platform of the Fondazione E. Mach): in brief, whole DNA was
129 extracted from 50 mg of faecal sample as described in Santa et al. 2019 [20], followed by qPCR
130 amplification (Viia 7 Real-time PCR System, ThermoFisher Scientific, Waltham, MA) targeting
131 the mitochondrial DNA marker *rrnL*, using 10 pmol of species-specific primers for *E.*
132 *multilocularis* (202 bp) and 0.2 pmol of hydrolysis probe. The qPCR-positive samples were
133 amplified with the same primer pair and sequenced with the dideoxy chain-termination
134 protocol on an ABI PRISM 3730xl Genetic Analyzer (Applied Biosystems) using the BigDye
135 Terminator cycle sequencing kit (Perkin Elmer, Applied Biosystems Division, Foster City, CA,
136 USA). Sequences were aligned with an *E. multilocularis* reference fragment (GenBank acc. n.
137 AB018440) using BioEdit 7.0.9 [21].

138 In order to evaluate the performances of the two diagnostic tests, a subsample of fox intestines, was
139 examined for adult worms by the Scraping, Filtration and Counting Technique (SFCT), considered as
140 the gold standard for *E. multilocularis* detection, following Santa et al. 2018 [22]. We performed SFCT
141 on the available small intestines of the foxes that had tested positive for one or both diagnostic tests

142 on faeces (n= 23), plus on a manageable number of fecal-negative samples (n=100), for a total of 123.
143 Briefly, the pylorus to caecum section of the small intestine was cut into 30 cm lengths, each of which
144 was then opened longitudinally and rinsed with tap water to remove loose faecal content. The
145 intestine was then scraped, the resulting wash water was poured through a series of sieves (1000
146 μm , 212 μm and 75 μm), and the respective filtrates were collected separately into three distinct
147 beakers. The filtrates were analysed by stereomicroscope (10X-63X) in 5 cm gridded petri dishes, to
148 count the adult helminths. Using the SFCT results, sensitivity (Se) and specificity (Sp) with 95% exact
149 binomial confidence intervals were calculated for each test, and their agreement (Cohen's Kappa) to
150 the standard was provided. True prevalence (TP) was then estimated, using the adjustment to the
151 Rogan-Gladen formula as proposed by Lang and Reiczigel for confidence intervals [23]. Finally, the
152 sample size needed for the detection of *E. multilocularis* by each of the two tests was assessed at the
153 estimated TP and according to varying expected prevalence using the modified binomial
154 approximation analysis method implemented in EpiTools
155 (<https://epitools.ausvet.com.au/freecalctwo>). We set a target population size of 300 individuals on a
156 local scale (hypothetical epidemiological unit), based on the results of unpublished data from wildlife
157 management offices in different areas of the northeastern Italian Alps, providing estimated densities
158 of 2.1, 3.4 and 7.5 foxes/km².

159

160 Results

161 The distribution of the 235 fox faecal samples and of the 123 fox small intestines is shown in in Fig 1.

162 Of the 235 fox faecal samples, seven (2.9%) were CMPCR-positive and 34 (14.4%) were qPCR-positive.

163

164 Fig 1 Distribution of individual fox faecal samples from Alto Adige in 2019-2020 (n=235) and subset
165 examined for adult worms by SFCT (n=123)

166 Adult *E. multilocularis* were found by SFCT in 24/123 (19.5%) fox small intestines (Fig 2) while, for the
167 same animals, five (4.1%) and 23 (18.7%) faecal samples resulted as CMPCR and qPCR positive,
168 respectively. Positive outcomes for the three diagnostics are summarized in Table 1.

169

170 Fig 2 Results of the fox fecal samples collected in 2019-2020 (n=123) tested by Coproscopy+Multiplex
171 PCR (CMPCR), qPCR and Scraping, Filtration and Counting Technique (SFCT- gold standard) for *E.*
172 *multilocularis* in Alto Adige, Italy

173

174 **Table 1 Matrix of the results of the two faecal tests (CMPCR and qPCR) and the gold standard**
175 **(SFCT) for the detection of *E. multilocularis* in 123 foxes**

Test	Coproscopy + Multiplex PCR (CMPCR)	qPCR	Scraping, filtration counting technique (SFCT)
CMPCR	5 (4.1%)		
qPCR	5	23(18.7%)	
SFCT	5	20	24 (19.5%)

176

177 Compared to SFCT, CMPCR detected 'true' infections in 5/24 (4.1%) cases, resulting in very poor
178 sensitivity (Se) (0.21; Table 2), and far lower than previously reported (about 0.55 [18]). Conversely,
179 qPCR confirmed the SFCT results in 20/24 (86.9%) of the cases, translating into a much higher Se
180 (0.83; Table 2). The qPCR test also identified three positive samples that were negative for SFCT, and
181 thus considered as false positives.

182

183 **Table 2 Sensitivity, specificity and agreement (K) for two diagnostic methods (Coproscopy+Multiplex**
184 **PCR [CMPCR] and qPCR) for detecting *E. multilocularis* in fox fecal samples compared to the Scraping,**
185 **Filtration and Counting Technique (SFCT - gold standard)**

Index	CMPCR versus SFCT (95% CI)	qPCR versus SFCT (95% CI)
Sensitivity (Se)	0.21 (0.07 - 0.42)	0.83 (0.63- 0.95)
Specificity (Sp)	1 (0.96 - 1)	0.97 (0.91 - 0.99)
Agreement (K)	0.30 (0.09 - 0.50)	0.82 (0.68 - 0.95)

186

187 Based on the Se and Sp calculated from these results (Table 2), the TP estimated for the whole sample
188 set (n=235) was 14.30 % (CI95%: 0-29.14) for CMPCR and 14.24% (CI95%: 7.04 -22.64) for qPCR. The
189 sample size required for the detection of *E. multilocularis* in a fox population of 300 individuals with
190 various expected prevalences is shown in Fig 3. For example, if the expected prevalence is 15%, about
191 90 individuals (about one third of the population) should be tested when using CMPCR, whereas
192 about 60 individuals (20%) would be enough to detect parasite presence by qPCR.

193

194 **Fig 3 Sample size required to detect *E. multilocularis* at various expected prevalences (with 95% CI)**
195 **in a population of 300 foxes, based on Sensitivity and Specificity of CMPCR and qPCR estimated here.**
196 **Regression was obtained by interpolating 13 prevalence values.**

197

198 Discussion

199 Our results lead to the conclusion that the true prevalence of *E. multilocularis* in Alto Adige has been
200 higher than previously reported in the last decade (<5%). According to the standards proposed by
201 Casulli et al. (2015), this region should now be considered as highly endemic (prevalence >10%) [24],
202 suggesting that surveillance should be intensified both locally and in the bordering territories.

203 The methods most widely used in the EU for detecting *E. multilocularis* in animal carcasses are
204 modifications of techniques targeting adult parasites, such as the sedimentation and counting (SCT)
205 and intestinal scraping (IST) [24]; the copro-antigen ELISA test [25]; or PCR [26] for species
206 determination. However, methods aimed at detecting adult helminths have practical limitations,
207 since they require the collection and necropsy of a consistent number of DHs throughout a study
208 area, storage capacity of large volumes of organs temporarily at -80°C (for ensuring positive samples
209 are not infective) and long-term at -20°C (for storage), as well as being time-consuming (at least 3
210 hours for each sample). Therefore, methods on faeces remain the best option for surveillance, given
211 also that faecal samples can be collected in the field using a standardized protocol, and many samples
212 can be screened simultaneously and rapidly. Surveillance coupling monitoring faeces of both fox
213 carcasses and scats would better encompass the life cycle of both the parasite and its main definitive
214 host throughout the year, and faecal prevalence could be used as a proxy for human risk of exposure.
215 In addition, non-invasive collection and genotyping of scats would allow repeated sampling on the
216 same fox social groups, tracking the seasonal pattern of infective eggs shedding at an individual level.
217 Antigenic and molecular tests are rapid and useful in regions characterized by a high parasitic
218 presence [27], but copro-antigen ELISA has the disadvantage of a low sensitivity, especially in the
219 case of low parasitic burden [24]: molecular methods remain therefore the best option. Based on our
220 results, tests directly applied on faeces, as qPCR, are recommended: in fact, besides reducing the
221 number of samples needed to detect the infection (Fig 3) and consequently, the sampling effort and
222 the cost of analyses, qPCR would also be the best option for detecting parasite DNA from scats
223 collected in the field, since these generally result in poor quality DNA compared to fresh fecal samples
224 taken during necropsies [28]. The higher sensitivity of qPCR is offset by an apparent decrease in
225 specificity, suggested by the three positive samples not identified by SFCT (see also [29]). However,
226 since SFCT is designed to detect adult worms, it may be that the extra positive samples detected by

227 qPCR were indications of late infections, when traces of parasite DNA may still be present in the
228 intestine but adult helminths are no longer macroscopically detectable or, on the contrary, of early
229 infections, when immature parasites (scolices) are present but are very difficult to identify
230 microscopically [14].

231 At the edges of *E. multilocularis* distribution, an efficient strategy for surveillance would be to
232 progressively include areas bordering the foci, in which the parasite could have gone undetected, or
233 in which *E. multilocularis* had been found occasionally, but no further confirmed, using qPCR as the
234 main diagnostic tool. In such a framework, we suggest that surveillance in foxes should be performed
235 on a small scale (e.g. considering an area similar to an Alpine valley as the basic epidemiological unit)
236 rather than a larger one (e.g. using the NUTS at level 1 or 2, as recommended for *E. granulosus* by
237 Tamarozzi et al. 2020 [30]). This would optimize efforts by increasing the chances of detecting *E.*
238 *multilocularis* in areas previously considered free from the parasite. Moreover, such a detailed
239 surveillance would be more informative of the actual risk of exposure for humans.

240 Finally, it is worth noting that a high endemicity of *E. multilocularis*, as that found in Alto Adige, should
241 be a mandate for increased surveillance of possible human cases, as well as infections in domestic
242 dogs. As dog infection could definitely increase the risk to humans, we suggest that this surveillance
243 also use the most sensitive molecular tools available.

244

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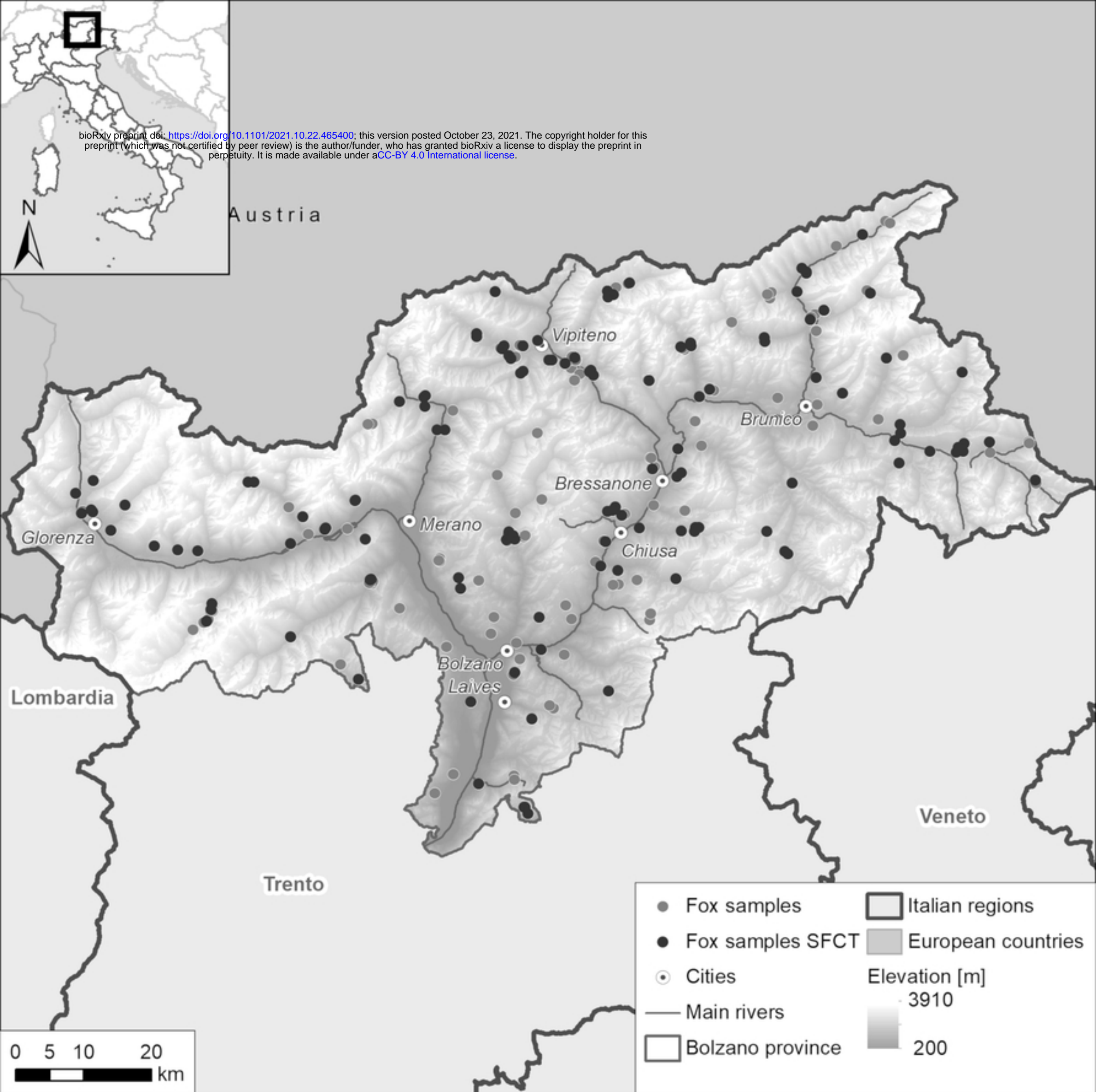


Fig1

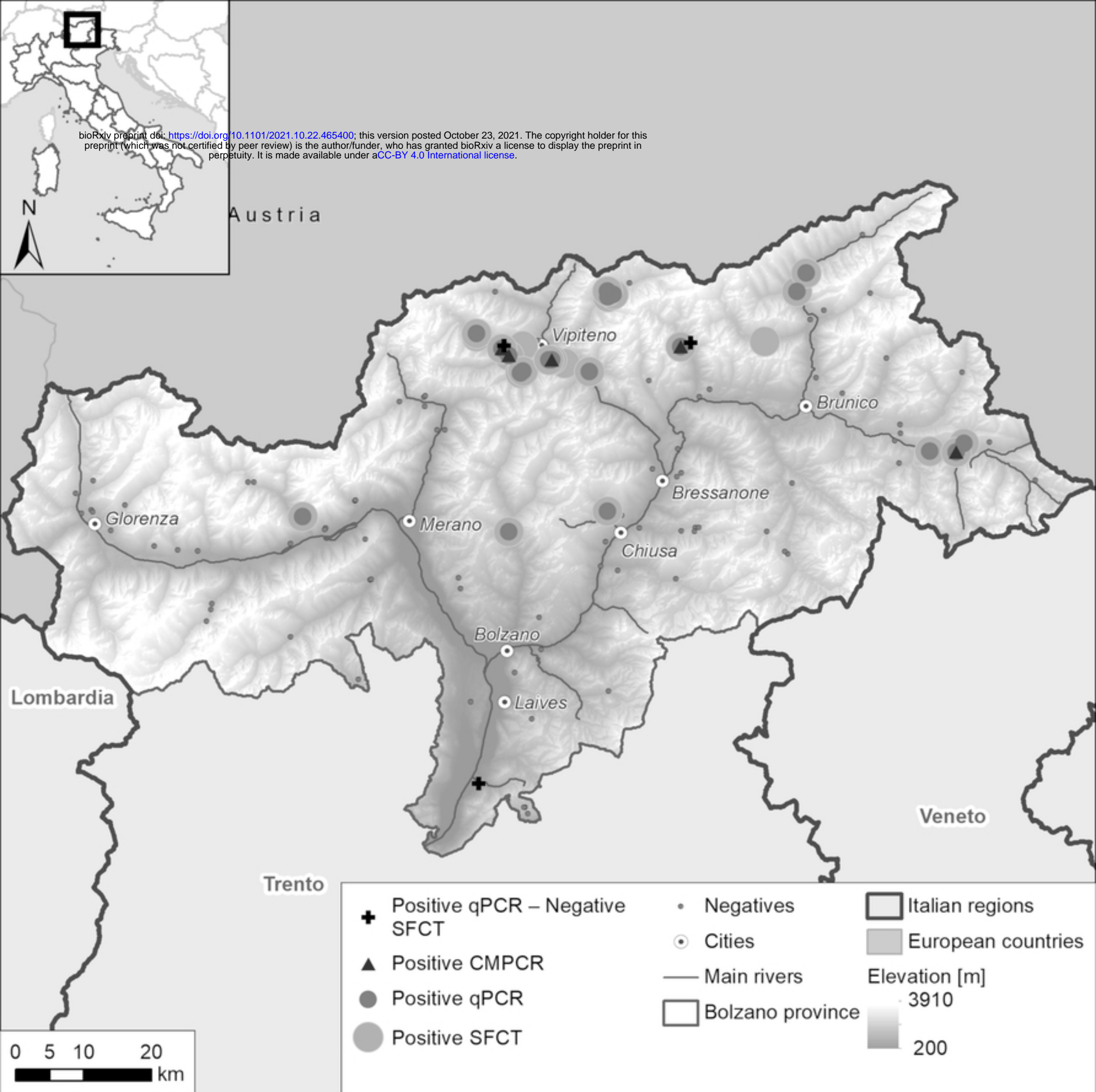


Fig2

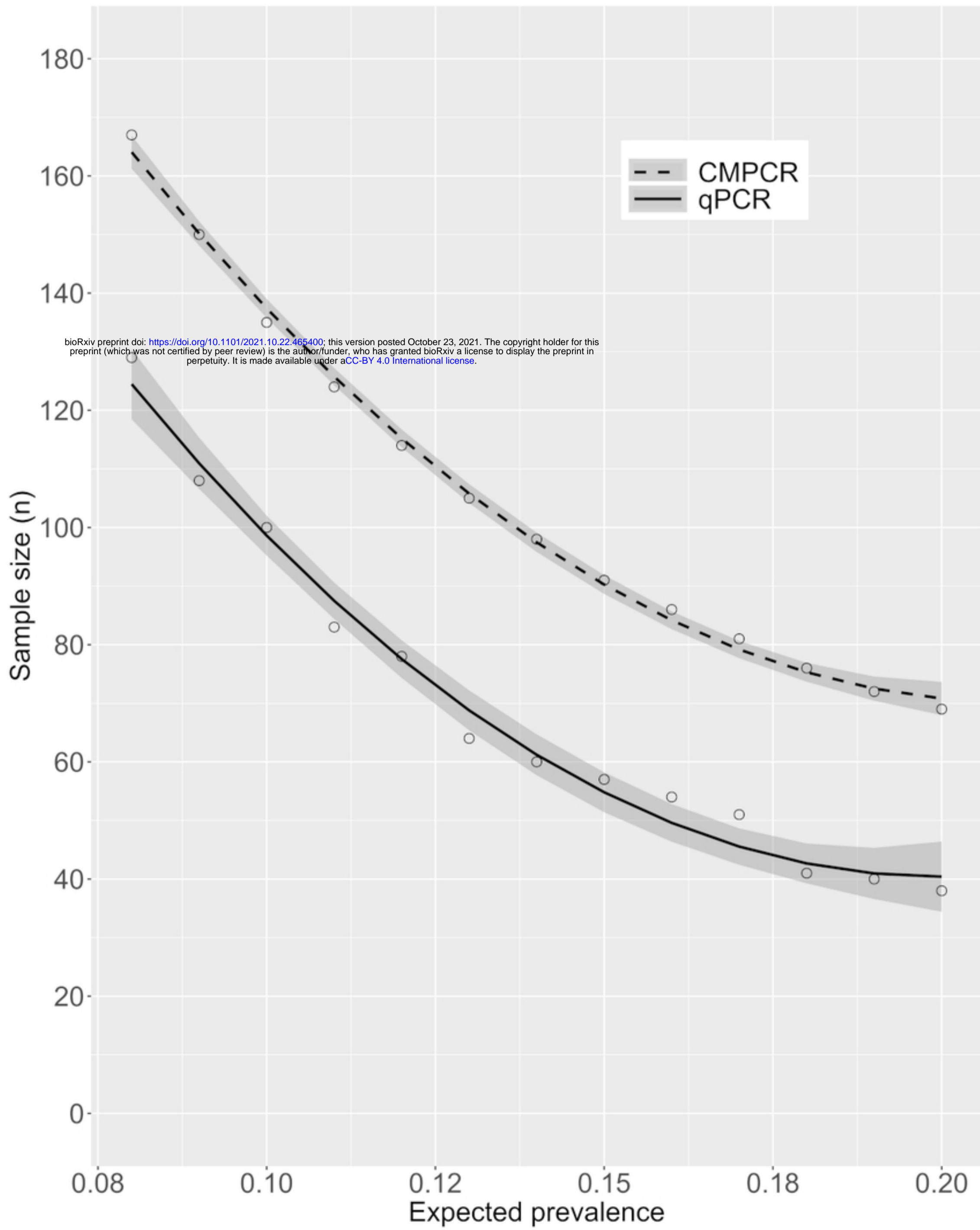


Fig3