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Characterisation of 'giant' cysts of the potato cyst nematode, *Globodera rostochiensis*, present in potato fields in Kenya

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Summary – Potato production in East Africa is seriously impacted by the potato cyst nematode (PCN), *Globodera rostochiensis*, where it has been recorded in at least three countries. In Kenya, it is widespread in all major potato-growing regions, often at very high densities. Consecutive cropping of potato on the same land and a sub-tropical climate have influenced PCN biology. For example, unusually large cysts have been regularly recovered. We have analysed the biological properties of these 'giant cysts'. The giant cysts contained more eggs than those recovered from UK fields. Egg size did not differ from UK populations and there was no difference in overall lipid content or lipid profile in J2 from giant cysts, compared to control samples. The nematodes in giant cysts were also genetically indistinguishable from any other *G. rostochiensis* sampled. When grown under UK glasshouse conditions, the offspring of nematodes from giant cysts were no different in size from those grown from control cysts, indicating that gigantism is not a heritable trait and may simply reflect favourable conditions for PCN under Kenyan farming systems. To date, all the PCN tested from Kenya, including those from giant cysts, are avirulent on potato cultivars containing the *H1* resistance gene.

Keywords – cyst biology, cyst DNA, cyst heritability, East Africa, nematode egg size, potato.

Potato cyst nematodes (PCN) originate from South America, where they co-evolved with their solanaceous hosts. PCN were imported to Europe in the mid-1800s (Evans *et al*., 1975), which then served as a secondary site of introduction to most potato-growing areas of the world. They are classified as quarantine pests in many regions and are amongst the most economically important pathogens of potatoes globally. Genetic studies of global PCN populations indicate that a relatively limited number of introductions of PCN into Europe have occurred (Blok *et al*., 1997; Hockland *et al*., 2012) and that distribution of PCN-contaminated seed from Europe has resulted in a further global spread of PCN (Hockland *et al*., 2012). In spite of extensive phytosanitary regulations and implementation of preventative measures, new infestations of PCN continue to be reported across the world. This includes the recent detection of one species of PCN, *Globodera rostochiensis*, from East Africa, in Kenya (Mwangi *et al*., 2015), Rwanda (Niragire *et al*., 2019) and Uganda (Cortada *et al*., 2020). Although *G. pallida* was also identified in Kenya (Mburu *et al*., 2018), detailed surveys found that this species is not widespread (Mburu *et al*., 2020).

Cropping systems in East Africa are markedly different to those in Europe. In Kenya, there is no seasonal winter under the sub-tropical climatic conditions. Many growers

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will plant two crops each year in conjunction with the rainy seasons, while continuous year-round cropping is possible where irrigation is available (Mburu *et al*., 2020). Crop rotation is poorly practised, and potato will often be planted repeatedly in the same fields in the key potato-growing areas, where smallholder farmers dominate. The most popular cultivar in Kenya, 'Shangi', grown by over 75% of smallholder farmers has no natural resistance to PCN. Consequently, extremely high populations of *G. rostochiensis* (up to 158 eggs (g soil)$^{-1}$) have developed in fields across many potato-growing areas in Kenya and are responsible for extensive yield losses (Mburu *et al*., 2020). In the absence of harsh winters, some Kenyan populations of PCN have lost their need for the overwintering diapause (Mwangi *et al*., 2021) that is necessary for survival in Europe. Moreover, anecdotal reports have indicated extremely large *G. rostochiensis* cysts originating from Kenyan fields (Kim Davie, SASA, pers. comm.). In order to determine the basis for such reports, we conducted a study of Kenyan *G. rostochiensis* cysts to characterise the biological properties of these 'giant' cysts. Such information is required to understand the biology of new geographic populations, towards developing informed PCN management strategies for the region.

Materials and methods

Biological material

Cysts were collected from three Kenyan field sites (Chairman, Kipipiri and Statehouse 0°40′57.792″S, 36°36′22.283″E; 0°18′54.068″N, 36°28′59.808″E; 0°39′02.2″S, 36°38′08.7″E, respectively) and compared with standard Scottish field population cysts collected from a heavily infested field in Angus (56°42′26.6″N 2°50′11.9″W). Extraction of cysts from soil was undertaken using standard flotation procedures and cysts were stored at 4°C until use. Eggs were extracted from cysts by dissection for further analysis. In order to obtain second-stage infective juvenile stage (J2) nematodes, cysts were immersed in tomato root diffusate (TRD) and J2 collected within 2 days of hatching (Price *et al*., 2023). Cysts, eggs and J2 were imaged using an OMAX A35180U3 microscope camera (AmScope) and their area quantified using ImageJ. A minimum of 100 cysts from each site were used for this analysis.

Lipid analysis

Oil Red O lipid staining and quantification was used to compare lipid content of juveniles from giant cysts with those from small cysts and from Scottish populations. The staining protocol described by Wang & Ching (2021) was used for this but modifications for use with PCN were made. Cysts and eggs were crushed to release J2 using a tissue homogeniser. The J2 were fixed in 2% paraformaldehyde in phosphate buffered saline overnight. Stained J2 were imaged using an OMAX A35180U3 microscope camera and staining was quantified in ImageJ. A minimum of 40 J2 were analysed in this way from each population. Total lipid extractions, made using the Bligh-Dyer technique (Bligh & Dyer, 1959) while vortexing overnight with glass beads, were dried under N_2 gas before adding 1.5 ml methanol, 0.2 ml toluene and 0.3 ml of 8% HCl in methanol:water (85:15) and then incubating overnight at 65°C to prepare fatty acid methyl-esters (FAMEs) for comparative analysis.

Genetic analysis

DNA was extracted from at least five single cysts from each of the populations being studied. For this, individual cysts were crushed in a plastic pestle in a 1.5 ml Eppendorf tube in 30 μl PCR Buffer. After a brief centrifugation to pellet debris, the supernatant was transferred to a fresh tube and heated to 95°C for 15 min before being cooled on ice. Proteinase K (Roche) (3 μg) was added to each sample, which was then incubated at 65°C for 2 h, followed by incubation at 95°C for 15 min. Samples were stored at −20°C until use. Diagnostic regions of the CytB and ITS regions were amplified using Phusion proof-reading polymerase (Thermo Fisher Scientific) with the primer pairs CytB_F (5′-GAAAAATTTTGGTAAT-3′) with CytB_R (5′-ATCATTTAACCCCTTTTTAG-3′) and ITS_F (5′-CGTAACAAGGTAGCTGTAG-3′) with ITS_R (5′-AGCGCAGACATGCCGCAA-3′). The presence of a single PCR product of the anticipated size was checked by agarose gel electrophoresis and PCR amplicons were cleaned by adding 2 μl of ExoSAP-IT (Thermo Fisher), incubating at 37°C for 15 min, followed by 15 min at 80°C to inactivate the enzyme. Products were sequenced in both directions on an ABI 377 sequencer at The James Hutton Institute sequencing facility using the primers indicated above.

Infection assays

The heritability of the giant cyst phenotype was assessed by inoculating each population on susceptible 'Desiree') plants under standard conditions. For this, potato tuber plugs bearing a chit were planted in deep root trainers (Haxnicks) filled with sterile sand/loam mix (50/50) into which approximately 15-20 cysts had previously been mixed. Plants were grown at 20°C under 16 h light/8 h dark conditions in a glasshouse for 10 weeks. Four replicates for each population were carried out and at least 40 of the resulting cysts that developed were extracted and measured as described above. In addition, virulence against the *H1* gene was assessed for each Kenyan population by assessing infection of the resistant 'Maris Piper' as described above.

Box plots and statistics

All box plots were created using the open source BoxPlotR webtool (Spitzer *et al*., 2014) (available at: http://shiny.chemgrid.org/boxplotr/). Analysis of variance was used to identify variance between multiple populations followed by *t*-tests to confirm significant differences between two population.

Results

Cyst, egg and juvenile measurements

Cysts present in Kenyan field samples showed a wide range of sizes and included cysts with a mean area of over 0.3 mm^2, which are referred to as giant cysts (Fig. 1A, B). Giant cysts were present in each of the three farms sampled (Chairman, Kipipiri and Statehouse). Analysis of the size distribution of the cysts showed significant differences between the size of giant and smaller cysts from each of the Kenyan farms and that these were all significantly different from cysts collected from the Scottish site (Fig. 1C) (*t*-test, $P < 0.01$). Notably, the 'small' cysts from Kenyan samples were significantly ($P < 0.01$) larger than those from Scottish fields. The number of eggs present in giant and small cysts from each site were compared. At each site giant cysts contained more eggs than small cysts although these differences were not always statistically significant (Fig. 2A). There was no difference in the size of the eggs from giant, small or Scottish cysts (Fig 2B). The J2 from giant cysts were slightly larger than those from smaller cysts but these differences were not statistically significant (Fig. 2C).

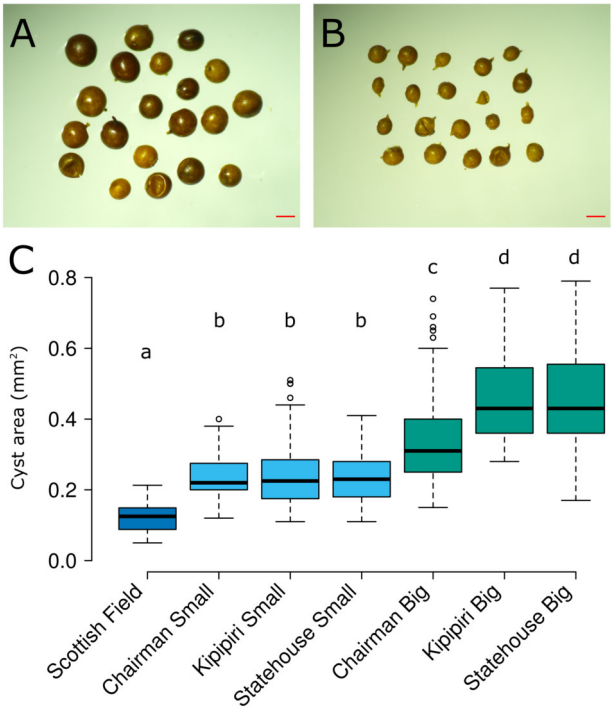

Fig. 1. Size comparison of giant (A) and smaller (B) Kenyan cysts of *Globodera rostochiensis* (PCN); All Kenyan cysts have a significantly larger area than typical PCN cysts from a Scottish field (C). Centre lines in boxplots show the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles, outliers are represented by dots. n = 100 sample points. There was no significant difference between the area of smaller Kenyan cysts taken from three locations, similarly, there was no significant difference between giant cysts from the Kipipiri and Statehouse farm sites. (Scale bar = 500 μm.)

Lipid content

Oil red O staining was used to measure the lipid content in J2 (Fig. 3A). The area of each stained J2 was then measured as a proxy for total lipid content. No significant differences between the lipid contents of J2 from Kenyan giant or small cysts or from the Scottish field population were observed (Fig. 3B) (ANOVA; $F < F_{critical}$).

GC-MS traces of fatty acid methyl-esters (FAMEs) extracted from each of the samples showed standard lipid abundance for PCN with large peaks for C18:1, C18:0, C20:1 and C20:0 and reduced C20:4. These data were similar to previous data for this species collected before exposure to hatching factors (Holz *et al*., 1998). There were no obvious differences in fatty acids between giant

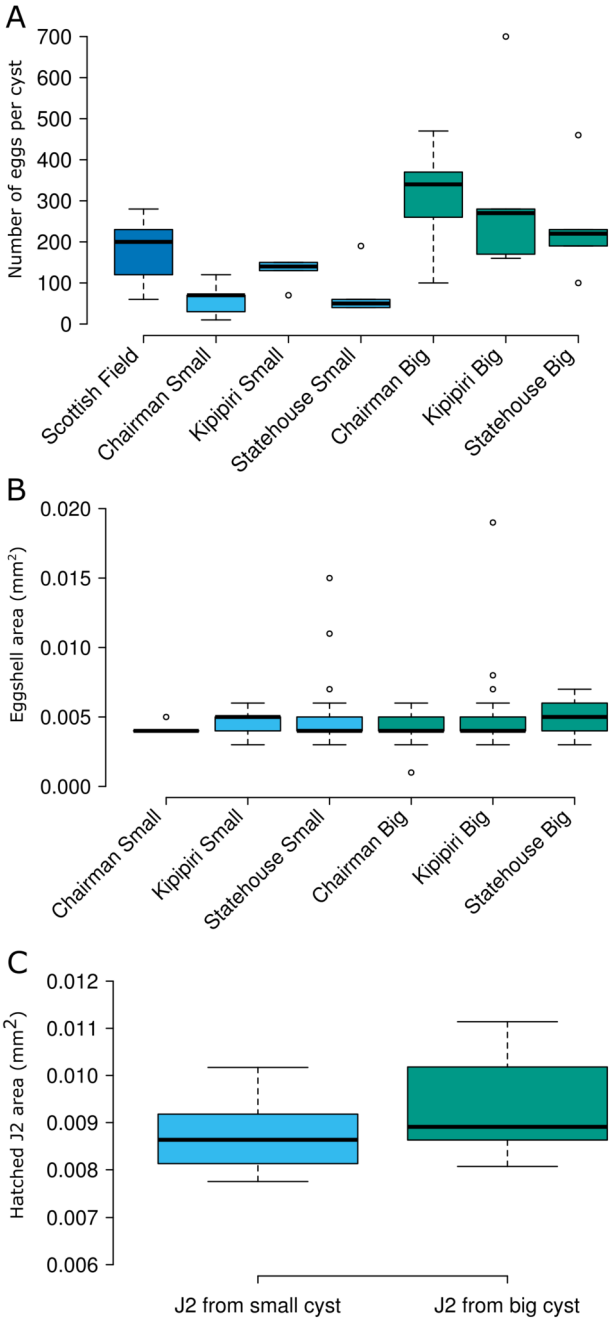

Fig. 2. Analysis of egg number (A), egg size (B) and J2 size (C) from giant and smaller cysts of *Globodera rostochiensis* from Kenyan field sites. Significantly greater numbers of eggs were present in giant cysts but there were no significant differences in size of eggs or second-stage juveniles (J2) from small and giant cysts.

and small cysts (Fig. 3C, D), with the same fatty acid species present in similar abundances.

Genetic analysis and heritability

Analysis of ITS and cytB sequences showed minimal differences (<2 bp) between giant and small cysts from Kenya or between Kenyan and Scottish samples (Suppl. Table S1 at 10.6084/m9.figshare.25965775). No structuring suggesting any genetic differentiation between the giant and small Kenyan PCN was observed.

When raised on susceptible plants in the glasshouse, the second generation of Kenyan *G. rostochiensis* from both small and giant cysts all developed to a similar size (Fig. 4). Notably, none of the second generation cysts that developed from giant cysts reached sizes similar to their maternal cysts. These data indicate that gigantism is not a heritable trait. None of the *G. rostochiensis* populations, whether giant or small Kenyan cysts, were able to reproduce on plants containing the *H1* resistance gene.

Discussion

Our study has established that under the sub-tropical climatic conditions in East Africa, *G. rostochiensis* naturally produces 'giant' cysts. Their mean fecundity and area are much larger than the Scottish comparatives used in the current study. Giant cysts were present at all three sites sampled in Kenya. Previous analysis of *G. rostochiensis* has shown that considerable variation exists in cyst size; Golden & Ellington (1972) reported cyst radii ranging from 125 μm to 405 μm, representing cyst areas from 0.05 mm^2 to 0.52 mm^{2,} with an average of 0.23 mm^2. While the majority of cysts measured for the present study fell within this range, there were numerous cysts from Kenyan fields that were larger than this. However, few biological differences were observed in the eggs or J2 from small or giant cysts, or indeed between Kenyan or Scottish populations. Eggs and J2 are similar in size, irrespective of whether they are derived from small or giant cysts, with no differences in total lipid content or lipid profile recorded. No genetic differentiation was discernible between small and giant cysts and infection studies showed that gigantism was not a heritable trait. All of these data suggest that giant cyst development in Kenya is due to the favourable environmental and agricultural conditions present. It is also likely that *G. rostochiensis* is thriving on the dominant cultivar used

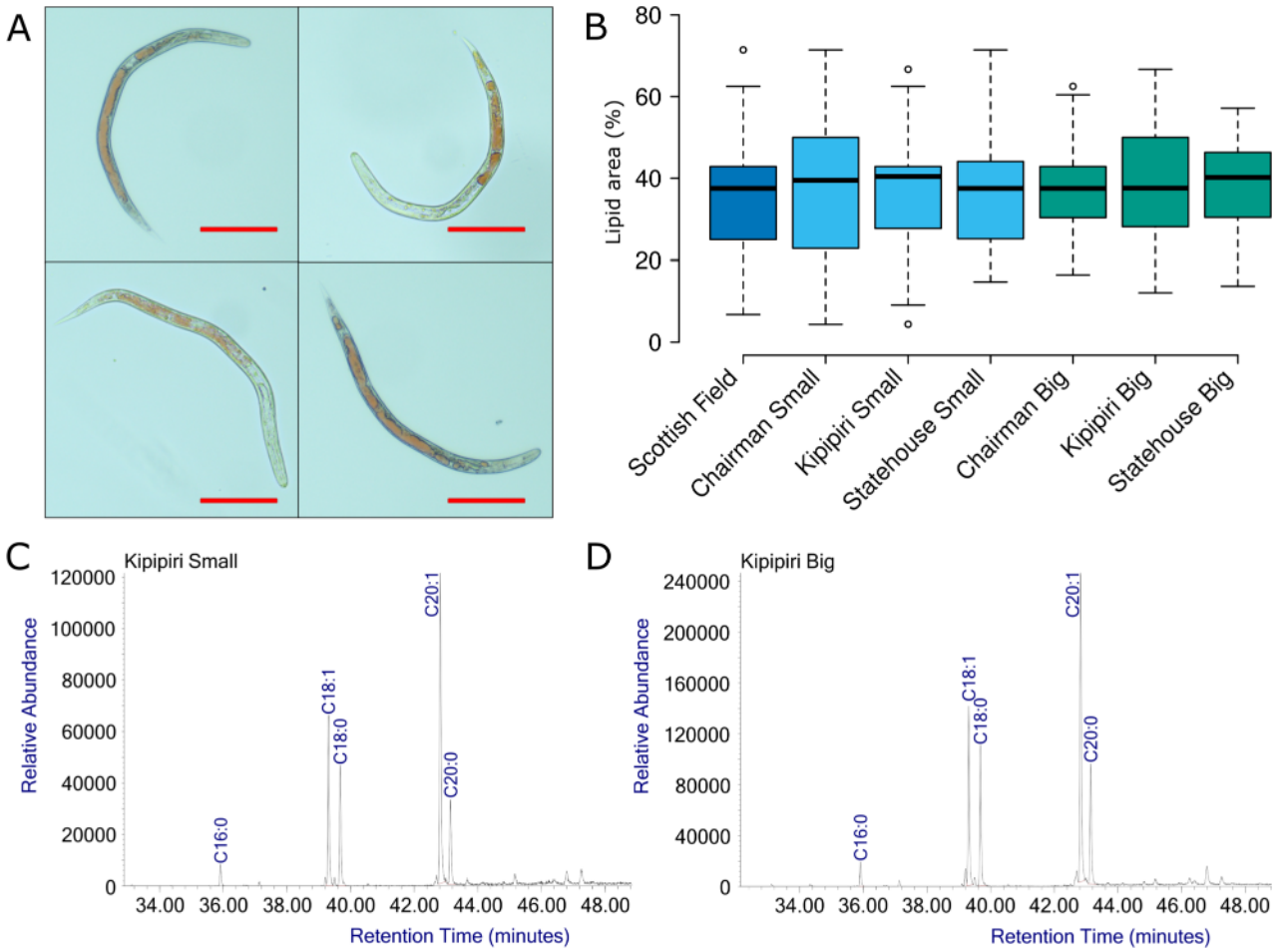

Fig. 3. Lipid analysis of second-stage juveniles (J2) from giant and small cysts of *Globodera rostochiensis*. A: lipids stained with oil red O in J2 from giant (top) and small (bottom) cysts; B: Comparison of stained lipid area *vs* total body area between different populations. Centre lines show the medians; box limits indicate the 25th and 75th percentiles as determined by R software (available at: http://shiny.chemgrid.org/boxplotr/); whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles, outliers are represented by dots. There was no significant difference between the quantity of stained lipids in juveniles from giant and small cysts. FAME analysis (GC-MS) shows there was no substantial diversity in fatty acid species in cysts from giant (C) or small (D) populations. Reduced FAME abundance in smaller cysts likely reflects a smaller number of eggs/J2 in these cysts as shown in Figure 2C. FAMEs identified did not differ from fatty acid species extracted from Scottish cysts.

by growers in the region, 'Shangi'. Although the genetic provenance of 'Shangi' is unclear, it is most likely a cultivar produced by The International Potato Centre (CIP) that was shared by growers after performing well in initial field trials (Thiele *et al*., 2021). The properties of 'Shangi' indicate that it likely has *Solanum phureja* in its genetic pedigree. 'Shangi' is a quick maturing variety with almost no dormancy that cooks extremely quickly and is highly favoured by farmers for these properties. In addition, tubers have a golden colour, suggesting high levels of carotenoids. It has been shown that transgenic potato lines in which the gene encoding carotenoid cleavage dioxygenase 4 (CCD4) was silenced by RNAi accumulated high levels of carotenoids (Campbell *et al*., 2010) and that significantly higher numbers of PCN developed on these lines compared to a control (Mei, 2015). 'Shangi' may therefore be an extremely favourable host for PCN.

Population studies of PCN have shown that the number of eggs produced per unit of haulm is negatively correlated with initial population density (Seinhorst, 1986,

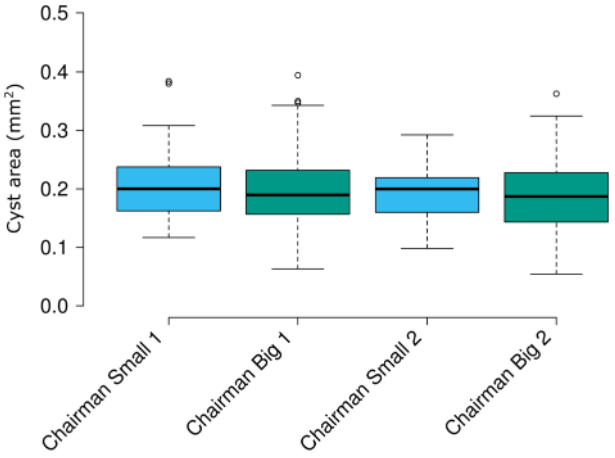

Fig. 4. Size comparison of the second generation of cysts of *Globodera rostochiensis* developing from small or giant cysts grown under controlled glasshouse conditions. Centre lines show the medians; box limits indicate the 25th and 75th percentiles as determined by R software (available at http://shiny.chemgrid.org/boxplotr/); whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles, outliers are represented by dots. There were no significant differences between second-generation cysts.

1993). However, while field densities are extremely high in Kenya, including at the sites sampled in the current study, this has not affected or prevented the development of giant cysts. It is likely that the conditions present in Kenya are currently strongly favourable for *G. rostochiensis* and that this nematode has adapted its biology to exploit these conditions, including through the production of giant cysts that contain substantial numbers of eggs.

Although *G. pallida* has been described in Kenya (Mburu *et al*., 2018), it was from a single site and despite a substantial nationwide survey it has not been further detected (Mburu *et al*., 2020). Several studies have shown that *G. rostochiensis* is better adapted to higher temperatures than *G. pallida* (*e.g.*, Robinson *et al*., 1987; Kaczmarek *et al*., 2014; Jones *et al*., 2017). Conditions in Kenya may therefore be more favourable for *G. rostochiensis* than for *G. pallida*. In addition, it has been shown that facultative diapause (as opposed to an obligate diapause) is more readily induced in *G. rostochiensis* than in *G. pallida* (*e.g.*, Salazar & Ritter, 1993). It is therefore possible that the ability to avoid diapause in *G. rostochiensis* has enabled it to exploit more readily the favourable conditions present in Kenya and outcompete *G. pallida*.

Effective tools for management of PCN throughout East Africa are urgently required. The deployment of plant host resistance against *G. rostochiensis* will form a critical component of any management strategy. Numerous cultivars are available in the region that contain the *H1* gene and such cultivars can provide effective control of the nematode. However, this resistance needs to be available in cultivars that contain the other traits preferred by growers if it is to be accepted and adopted and used as a management tool. Current cropping systems coupled with the adaptations shown by *G. rostochiensis* in the region, including the ability to produce extremely fecund giant cysts, mean that the levels of the nematode present in the region are likely to continue to increase and cause extensive yield losses.

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Supplementary Material

Supplementary Table S1. Total data from cysts sampled from a Scottish (UK) farm and samples from three farms in Kenya (Chairman, Kipipiri and Statehouse). A: Cyst area (mm^2); B: Egg number; C: Egg area (mm^2); D: Second-stage juvenile (J2) area (mm^2); E: Lipid area (as a percentage of the total J2 area); F: Cyst area, second generation (mm^2); G: Sequencing information. This table can be accessed at 10.6084/m9.figshare.25965775.

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