First report of the invasive golden apple snail, *Pomacea canaliculata* in Kenya

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17

18 **Abstract**

19 Following reports of an invasive snail causing crop damage in the expansive Mwea irrigation
20 scheme in Kenya, samples of snails and associated egg masses were collected and sent to
21 CABI laboratories in the UK for molecular identification. DNA barcoding analyses using the
22 cytochrome oxidase subunit I gene confirmed the identity of the snails as *Pomacea*
23 *canaliculata*, widely considered to be one of the most invasive invertebrates of waterways
24 and irrigation systems worldwide. To the best of our knowledge, this is the first record of *P.*
25 *canaliculata* in Kenya, and the first confirmed record of an established population in
26 continental Africa. This timely identification shows the benefit of molecular identification
27 when combined with a reliable database such as that provided by the Barcoding of Life Data
28 system. We found that the egg masses tested gave an identical barcode sequence to the
29 adult snails, allowing identifications to be made more rapidly. Given the impact of this
30 species in Asia, there is need for an assessment of the risk to Africa, and the implementation
31 of an appropriate response in Kenya and elsewhere to manage this new threat to agriculture
32 and the environment.

33

34 **Keywords**

35 Golden apple snail, invasive species, molecular identification, DNA barcoding, COI gene,
36 phytosanitary risk

37

38 **Background**

Apple snails (Ampullariidae) are freshwater gastropods distributed naturally throughout the humid tropics and subtropics (Berthold, 1991). *Pomacea*, the largest of nine extant genera in the family, are New World species native to South, Central and North America (Hayes et al. 2015). Some *Pomacea* species, mainly from South America, demonstrate notable invasiveness outside of their native ranges (Hayes et al. 2008; Wu et al. 2010; Lv et al. 2013; Yang et al. 2018). Of these, *Pomacea canaliculata* (Lamarck 1829), is listed among ‘100 of the world’s worst invasive species’ (Lowe et al. 2000) and together with the morphologically similar *P. maculata* (= *P. insularum*) (Perry 1910), have become serious agricultural and ecological pests, causing significant economic losses in wetland rice cultivation and threatening biodiversity (Cowie 2002; Tamburi and Martín 2009; Qiu et al. 2011). Historically, descriptions of *Pomacea* have been based on the conchological characteristics of a few specimens, resulting in taxonomic confusion (Cowie et al. 2006). Furthermore, identification and differentiation between species has proved extremely difficult (Thiengo et al. 1993; Cazzaniga 2002; Cowie et al. 2006) since morphological similarities are shared among many different species and they also exhibit substantial intraspecific and geographic variation (Hayes et al. 2012; Mahilum and Demayo 2014). In light of this, phylogenetic analyses have provided an invaluable framework for discovering and delineating apple snail species (Rawlings et al. 2007; Hayes et al. 2008; Yang and Yu 2019) and by correctly identifying species, pest and natural resource managers are provided with a better scientific foundation on which to implement effective management plans to mitigate spread, and minimize their agricultural and environmental impacts. Apple snails continue to spread worldwide and have been newly reported in Europe (López et al. 2010; EFSA 2020) and Ecuador (Horgan et al. 2014), but the African continent remained hitherto free of established populations (Seuffert and Martin 2017).

Methods

The aim of the study was to determine the identity of the snails found at two locations (Teberere and Ndekia) in Mwea irrigation scheme in Kenya and to determine if they were indigenous or a potentially invasive species.

Sample collection and handling:

Field surveys for apple snails were carried out in Kenya in September 2020 after reports that an unknown snail species had invaded close to 222 ha of Teberere and Ndekia section of the 10,117ha Mwea irrigation scheme in Kirinyaga County. Sampling sites included irrigated rice fields and other host plants at the scheme. At each sampling location, latitude, longitude and altitude were determined using a global positioning system device, Garmin, eTrek© 20x (Garmin, USA) (Figure 1). At each sample collection site, photographs were taken of the eggs (e.g. Figure 2a), adults (e.g. Figure 2b) and damage to rice crop (e.g. Figure 2c). Samples of eggs and adults were also collected for morphological and molecular identification. In total, 39 samples were collected from the two affected locations. Preliminary morphological identification to genus level was done using features described by Hayes et al. (2012). Adult snails were collected in plastic containers together with soil and water which were properly sealed to avoid spillage. Egg masses were collected together with plant part to which they were attached to avoid early crushing/breaking. A sample of the collected snails was rinsed in running tap water to remove soil and other debris, then transferred to clean containers and immersed in absolute ethanol (>99% v/v). The lids of the containers were tightened and the containers were stored in a laboratory refrigerator (at 2-8 °C). Egg masses were also collected and treated in the same way.

88

89 Figure 1: Map of Kenya showing survey locations in Mwea Irrigation scheme.

90

Figure 2: Examples of: a) snail egg masses; b) adult snail; c) rice crop damage; recorded within the Mwea Irrigation scheme, Kenya.

Of the samples collected, 15 adult snails and two egg masses were prepared for despatch to the UK. During packing, the ethanol was drained and the samples placed on dry cotton wool in a plastic container for shipment to CABI's diagnostic and molecular biology laboratories in the UK.

On receipt in the UK, samples were processed as follows:

101 Specimens were unpacked, subjected to visual examination and photographed. Three adult
102 snails, broadly covering the range of the sizes seen but otherwise selected at random, were
103 selected for identification plus one sample of an egg mass. Snail bodies were removed
104 carefully from the shells, using alcohol-sterilised, then flamed, forceps and immersed in
105 absolute ethanol (>99% v/v) in individual sterile tubes. The selected egg mass was
106 immersed in absolute ethanol (>99% v/v) in a separate tube. All tubes were transferred to a
107 laboratory freezer (-20 °C) in which they were stored for 48 hours, to ensure no living
108 material remained.

109 On retrieval from the freezer, several small sections of each snail sample were removed
110 from 'clean' areas (foot and the tentacles), using alcohol-sterilised fine forceps and scalpel,
111 taking care to avoid the digestive system and other organs. One egg was removed for
112 processing. These 'sub-samples' were placed in fresh sterile 1.5ml microcentrifuge tubes
113 containing absolute ethanol (>99% v/v) and placed in the freezer until they could be
114 processed further.

115

116 *Molecular methodology:*

117 Tubes containing snail samples were removed from the freezer for processing. Samples
118 were removed from the tubes and gently blotted dry on clean lint-free tissue paper. The
119 samples were then subjected to DNA extraction using the DNeasy Blood and Tissue kit
120 (Qiagen, UK) following the manufacturer's protocol, amended to incorporate an overnight
121 incubation at 55 °C once the buffer ATL and proteinase K had been added to the tissue
122 (Polaszek et al. 2014). Subsequent steps were as prescribed by the manufacturer.

123 PCR reactions were carried out using a Hybaid PCR Express thermal cycler in heated-lid
124 mode. Amplifications were carried out in 0.5 ml microcentrifuge tubes in 20 µl reactions
125 containing: 1 µl DNA extract; Primers LCO1490 and HCO2198 (5'-

GGTCAACAAATCATAAAGATATTGG-3' and 5'-TAAACTTCAGGGTGACCAAAAAATCA-3', respectively; Folmer et al. 1994) each at 150 nM; and 10 µl of MegaMix-Royal (Microzone Ltd, UK) mastermix solution, containing optimised mixture of *Taq* polymerase in 2 × Enhancing Buffer (6 mM MgCl₂), with 400 µM dNTPs and blue MiZN loading dye. Reactions were made up to a final volume of 20 µl with sterile molecular grade H₂O. PCR reactions were preincubated for 5 min at 95 °C followed by 39 cycles of: 30s at 94 °C; 30s at 51 °C and 75s at 72 °C. Samples were finally incubated for 10 min at 72 °C, then cooled and finally held at 10 °C for 1 hour before PCR products were visualised with gel electrophoresis, purified by microCLEAN (Microzone Ltd., UK), and resuspended in 15 µl sterile molecular grade H₂O. After sequencing reactions, excess unincorporated dye terminators were removed by means of DyeEx 2.0 (Qiagen, UK) gel filtration columns, according to the manufacturer's instructions. Eluted samples were resuspended in 16 µl highly deionised formamide (HiDi™; ThermoFisher Scientific, UK). Sanger sequencing was undertaken using an ABI 3130 Genetic Analyser (ThermoFisher Scientific, UK) in accordance with the manufacturer's instructions. Sequence trace files were examined for quality using the DNA Sequencing Analysis Software 6 (Applied Biosystems, UK), then exported as text files.

Data analysis:

Sequences obtained in the present study were screened against the holdings of NCBI-GenBank and EMBL-EBI using the BLAST (Altschul et al. 1990; Altschul et al. 1997) and FASTA (Pearson, 1990) algorithms, respectively. Sequences were then compared with authenticated sequences obtained from the Barcoding of Life Data system (BOLD; <http://www.boldsystems.org/>; Ratnasingham and Hebert, 2007) and additional sequences from the GenBank® data base (<http://www.ncbi.nlm.nih.gov/genbank/>). Sequences were aligned using the multiple sequence alignment tools CLUSTALW (Thompson et al. 1994)

and MUSCLE (Edgar 2004a,b) in MEGA7 (Kumar et al. 2016) and these were then optimized manually in the MEGA7 program. Evolutionary history was inferred by means of the maximum likelihood method based on the Tamura–Nei model (Tamura and Nei 1993). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor–Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach, before selecting the topology with superior log likelihood value. Codon positions included were 1st +2nd +3rd + noncoding whilst all positions containing gaps and missing data were eliminated. Trees obtained were drawn to scale, with branch lengths measured in the number of substitutions per site. The bootstrap consensus tree was inferred from 1000 replicates and is taken to represent the evolutionary history of the taxa analysed (Felsenstein 1985). Branches corresponding to partitions reproduced in less than 50% of the bootstrap replicates are collapsed. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the respective branches (Felsenstein 1985). Further evolutionary analyses were conducted in MEGA7 (Kumar et al. 2016)

Results

The four samples processed for sequencing (three adult snails plus one egg from an egg mass) gave identical sequences when barcoded with the COI primers. When screened against the holdings of GenBank and BOLD, all top matches, showing >99% identity to the Mwea samples, were to sequences assigned to *P. canaliculata* with the sole exception of what appeared to be an aberrant *P. maculata* sequence (MK992483) from Uruguay (data not shown). This sequence showed in the GenBank/BLAST and BOLD results but did not feature in the EMBL-EBI/FASTA top 500 results. This particular sequence also appeared confounding in the phylogenetic analysis undertaken subsequently as it was placed within the *P. canaliculata* cluster and not with the rest of *P. maculata*, which grouped with *P. insularum* (Figure 3). This is to be expected given that the name *Pomacea insularum* was formerly used as the valid name of *P. maculata* but is now a junior objective synonym of *P. maculata*, following the designation of a single specimen as both the neotype of *P. maculata* and lectotype of *P. insularum*; the same specimen was also designated as the neotype of *P. gigas*, thereby making this also a junior objective synonym of *P. maculata* (Hayes et al. 2012).

The evolutionary history of the sequences was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). The tree with the highest log likelihood (-6485.26) is shown as Figure 3. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 71 nucleotide sequences. All positions containing gaps and missing data were eliminated. In total, there were 546 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al. 2016).

These results show clearly that the samples tested are *P. canaliculata*. Sequences have been deposited in GenBank with the accession numbers MW363565-MW363568.

Discussion

Early detection of non-native invasive species is a critical factor to inform timely implementation of contingency and control measures and allow the greatest chance of successful management or eradication. The gradual erosion in morpho-taxonomic expertise worldwide, as well as the potential for confusion caused by taxa that look almost identical, such as the species of *Pomacea* (Estebenet et al. 2006), can make positive identification a challenge (Rama Rao 2018). Molecular approaches have contributed to improved taxonomic understanding and clarification of the distribution and invasion pathways of

208 *Pomacea* species (Kannan et al. 2020) and provide an invaluable and complementary
209 diagnostic tool for the timely identification of these highly destructive invasive species.

210 This appears to be the first confirmed report of *P. canaliculata* in continental Africa, although
211 it is present in La Réunion and Mauritius (EPPO 2020). Cowie et al. (2017) cite Wu and Xie
212 (2006) as an unconfirmed report of its presence in Egypt, and they also cite Berthold (1991)
213 reporting its presence in South Africa, noting that it was identified as *P. lineata* but was
214 probably *P. canaliculata*, and that it was possibly not established.

215 The arrival of this notorious invasive species in mainland Africa raises many questions that
216 cannot be dealt with in detail in this short report. One is how might it have arrived in Kenya?
217 In Asia it is thought to have been illegally but intentionally introduced to Taiwan around 1980,
218 with subsequent rapid spread to other countries in South-East Asia “predominantly human-
219 mediated” (Cowie 2002), often because it was seen as a potential food source. Unconfirmed
220 media reports in Kenya suggest that the snail was introduced to control weeds, but no permit
221 to import the species has been issued by the Kenya Standing Technical Committee on
222 Imports and Exports.

223

224 In South-East Asia the golden apple snail has caused significant crop losses in rice, resulting
225 in annual management costs of more than \$1200 million (Brito and Joshi 2016). In Kenya
226 farmers are already complaining about the damage caused to rice in Mwea, where over 70%
227 of the country’s rice is grown (Atera et al. 2018). In the initial area of infestation, farmers
228 have reported up to 92% damage on newly transplanted rice seedlings. Experience in Asia
229 indicates that damage decreases as seedling age increases and is higher where direct
230 seeding is used rather than transplanting seedlings (CABI 2020). *P. canaliculata* also

231 displays developmental and reproductive plasticity allowing adaptation to a wide range of
232 habitats with different climates and the magnitude of its environmental and agricultural
233 damage is climatically linked and predicted to increase globally (Lei et al. 2017).

234 How far might it have already spread in Kenya? Delimiting surveys are in progress, but the
235 Mwea rice production area drains into the Tana River, a designated Ramsar wetland site
236 and an important ecosystem in East Africa in which *P. canaliculata* could have significant
237 impacts. Although renowned for the damage it causes to agricultural production, there are
238 many actual and potential environmental impacts of *P. canaliculata* (Carlsson 2017; Martin
239 et al. 2019) including direct effects through herbivory, competition and predation, but also
240 indirect effects such as transmission of human disease and bioaccumulation of pollutants.

241 Having reached mainland Africa, there must also be concern that the snail will be
242 transported to other countries in the continent, including West Africa where nearly two-thirds
243 of Africa's rice is produced (Sers and Mughal 2020). Rice consumption in Africa is
244 increasing, and although the gap has been reduced, the continent is still unable to produce
245 sufficient rice to meet demand (Senthilkumar et al. 2020). No environmental suitability
246 modelling for *P. canaliculata* in Africa has been conducted, although models have been
247 constructed elsewhere that could be used to examine the potential spread (Gilioli et al.
248 2017). A risk assessment for *P. canaliculata* in Africa is needed as a priority for which such
249 modelling would be a useful input.

250

251 In the meantime, plans for containment measures and management should be a priority in
252 the sections/area already infested, in order to prevent spread. Further, the National Plant
253 Protection Organisations in Africa should be on the alert for this species. Based on a risk

assessment, phytosanitary measures should be implemented to reduce the likelihood of its introduction, but at the same time contingency plans should be prepared so that it can be detected and responded to early and effectively should it reach other countries.

Conclusions

The results obtained from our barcoding analysis show clearly that *P. canaliculata* is present in Kenya. Given this snail's ongoing reputation as one of the top 100 invasive species threats worldwide (Lowe et al. 2000), such an early finding has real significance for plant health in Kenya requiring urgent remedial action.

We have also clearly demonstrated the benefits of DNA barcoding for detecting and verifying such organisms in the absence of expert morphometric malacologists due to the benefits of the global sequence repositories such as BOLD. The matching results obtained from the egg showed that a barcoding approach enables significant time savings as there is no need to grow the egg to maturity for definitive identification.

List of abbreviations

BLAST = Basic local alignment search tool

BOLD = Barcoding of Life Datasystems

COI = Cytochrome oxidase c subunit I gene

dNTPs = deoxyribonucleotide triphosphates

276 EBI = European Bioinformatics Institute
277 EMBL = European Molecular Biology Laboratory
278 NCBI = National Center for Biotechnology Information
279 PCR = Polymerase chain reaction

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281

282 **Declarations**

283 ***Ethics approval and consent to participate***

284 Not applicable.

285 ***Consent for publication***

286 Not applicable.

287 ***Availability of data and materials***

288 The datasets used and/or analysed during the current study are available from the
289 corresponding author on reasonable request.

290 **Competing interests**

291 The authors declare that they have no competing interests.

292

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304 ***Authors' contributions***

305 FM, DC and KV undertook the initial survey and provided detail on the survey methodology;
306 LO did the laboratory analysis of the snail material in the UK and provided the
307 methodological input; GC undertook the molecular processing for identification and supplied
308 the technical information. AB developed the concept, undertook the phylogenetic analyses
309 and wrote the first draft. DD provided the background detail on the invasive species. IR and
310 RD provided funding and supervised the study. AB, DD, IR, AM, SK and RD contributed
311 further edits. All authors read and approved the final manuscript.

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447 Figure 3: Molecular phylogenetic analysis, by Maximum Likelihood method, of representative
448 *Pomacea* COI sequences (including those from the present study MW363565-MW363568).
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