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

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## Abstract

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



## Keywords

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


## 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 (Tebere 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 Tebere 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.

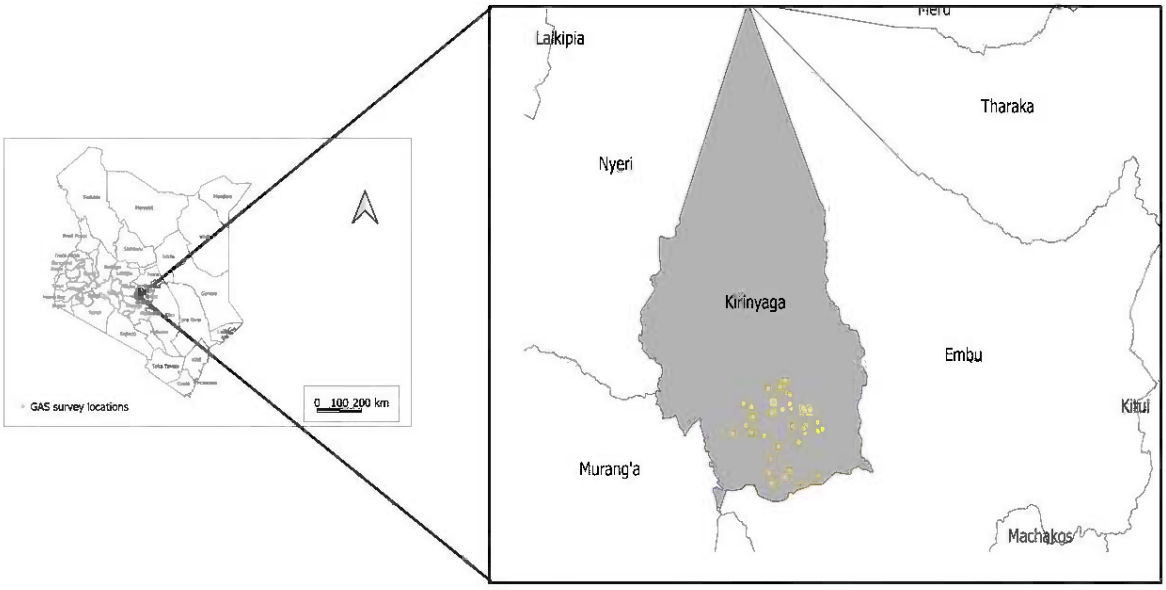


Figure 1: Map of Kenya showing survey locations in Mwea Irrigation scheme.


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:

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

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

*Molecular methodology*:

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

PCR reactions were carried out using a Hybaid PCR Express thermal cycler in heated-lid mode. Amplifications were carried out in 0.5 ml microcentrifuge tubes in 20 µl reactions 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 $MgCl_2$), with 400 µM dNTPs and blue MiZN loading dye. Reactions were made up to a final volume of 20 µl with sterile molecular grade $H_2O$. 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 $H_2O$. 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 $1^{st}$ +$2^{nd}$ +$3^{rd}$ + 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

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

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

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

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

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

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

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

In the meantime, plans for containment measures and management should be a priority in the sections/area already infested, in order to prevent spread. Further, the National Plant 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

EBI = European Bioinformatics Institute

EMBL = European Molecular Biology Laboratory

NCBI = National Center for Biotechnology Information

PCR = Polymerase chain reaction

## Declarations

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

Not applicable.

***Consent for publication***

Not applicable.

***Availability of data and materials***

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

**Competing interests**

The authors declare that they have no competing interests.

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### *Authors' contributions*

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

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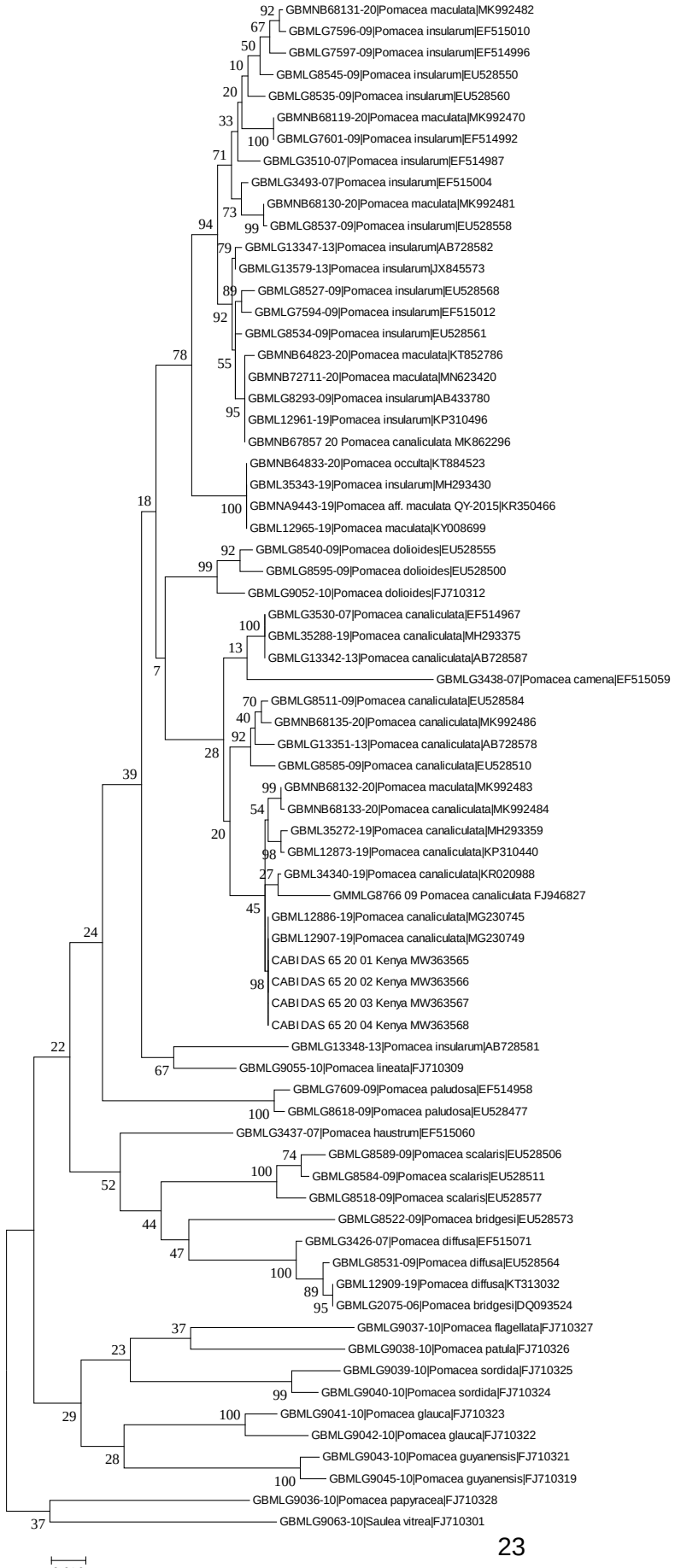
447 Figure 3: Molecular phylogenetic analysis, by Maximum Likelihood method, of representative

448 *Pomacea* COI sequences (including those from the present study MW363565-MW363568).

449


GBMNB68131-20|Pomacea maculata|MK992482
GBMLG7596-09|Pomacea insularum|EF515010
GBMLG7597-09|Pomacea insularum|EF514996
GBMLG8545-09|Pomacea insularum|EU528550
GBMLG8535-09|Pomacea insularum|EU528560
GBMNB68119-20|Pomacea maculata|MK992470
GBMLG7601-09|Pomacea insularum|EF514992
GBMLG3510-07|Pomacea insularum|EF514987
GBMLG3493-07|Pomacea insularum|EF515004
GBMNB68130-20|Pomacea maculata|MK992481
GBMLG8537-09|Pomacea insularum|EU528558
GBMLG13347-13|Pomacea insularum|AB728582
GBMLG13579-13|Pomacea insularum|JX845573
GBMLG8527-09|Pomacea insularum|EU528568
GBMLG7594-09|Pomacea insularum|EF515012
GBMLG8534-09|Pomacea insularum|EU528561
GBMNB64823-20|Pomacea maculata|KT852786
GBMNB72711-20|Pomacea maculata|MN623420
GBMLG8293-09|Pomacea insularum|AB433780
GBML12961-19|Pomacea insularum|KP310496
GBMNB67857 20 Pomacea canaliculata MK862296
GBMNB64833-20|Pomacea occulta|KT884523
GBML35343-19|Pomacea insularum|MH293430
GBMNA9443-19|Pomacea aff. maculata QY-2015|KR350466
GBML12965-19|Pomacea maculata|KY008699
GBMLG8540-09|Pomacea dolioides|EU528555
GBMLG8595-09|Pomacea dolioides|EU528500
GBMLG9052-10|Pomacea dolioides|FJ710312
GBMLG3530-07|Pomacea canaliculata|EF514967
GBML35288-19|Pomacea canaliculata|MH293375
GBMLG13342-13|Pomacea canaliculata|AB728587
GBMLG3438-07|Pomacea camena|EF515059
GBMLG8511-09|Pomacea canaliculata|EU528584
GBMNB68135-20|Pomacea canaliculata|MK992486
GBMLG13351-13|Pomacea canaliculata|AB728578
GBMLG8585-09|Pomacea canaliculata|EU528510
GBMNB68132-20|Pomacea maculata|MK992483
GBMNB68133-20|Pomacea canaliculata|MK992484
GBML35272-19|Pomacea canaliculata|MH293359
GBML12873-19|Pomacea canaliculata|KP310440
GBML34340-19|Pomacea canaliculata|KR020988
GMMLG8766 09 Pomacea canaliculata FJ946827
GBML12886-19|Pomacea canaliculata|MG230745
GBML12907-19|Pomacea canaliculata|MG230749
CABI DAS 65 20 01 Kenya MW363565
CABI DAS 65 20 02 Kenya MW363566
CABI DAS 65 20 03 Kenya MW363567
CABI DAS 65 20 04 Kenya MW363568
GBMLG13348-13|Pomacea insularum|AB728581
GBMLG9055-10|Pomacea lineata|FJ710309
GBMLG7609-09|Pomacea paludosa|EF514958
GBMLG8618-09|Pomacea paludosa|EU528477
GBMLG3437-07|Pomacea haustrum|EF515060
GBMLG8589-09|Pomacea scalaris|EU528506
GBMLG8584-09|Pomacea scalaris|EU528511
GBMLG8518-09|Pomacea scalaris|EU528577
GBMLG8522-09|Pomacea bridgesi|EU528573
GBMLG3426-07|Pomacea diffusa|EF515071
GBMLG8531-09|Pomacea diffusa|EU528564
GBML12909-19|Pomacea diffusa|KT313032
GBMLG2075-06|Pomacea bridgesi|DQ093524
GBMLG9037-10|Pomacea flagellata|FJ710327
GBMLG9038-10|Pomacea patula|FJ710326
GBMLG9039-10|Pomacea sordida|FJ710325
GBMLG9040-10|Pomacea sordida|FJ710324
GBMLG9041-10|Pomacea glauca|FJ710323
GBMLG9042-10|Pomacea glauca|FJ710322
GBMLG9043-10|Pomacea guyanensis|FJ710321
GBMLG9045-10|Pomacea guyanensis|FJ710319
GBMLG9036-10|Pomacea papyracea|FJ710328
GBMLG9063-10|Saulea vitrea|FJ710301
0.020



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