**Fusarium** species isolated from post-hatchling loggerhead sea turtles (*Caretta caretta*) in South Africa

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Species in the *Fusarium solani* species complex are fast growing, environmental saprophytic fungi. Members of this genus are filamentous fungi with a wide geographical distribution. *Fusarium keratoplasticum* and *F. falciforme* have previously been isolated from sea turtle nests and have been associated with high egg mortality rates. Skin lesions were observed in a number of stranded, post-hatchling loggerhead sea turtles (*Caretta caretta*) in a rehabilitation facility in South Africa. Fungal hyphae were observed in epidermal scrapes of affected turtles and were isolated. The aim of this study was to characterise the *Fusarium* species that were isolated from these post-hatchling loggerhead sea turtles (*Caretta caretta*) that washed up on beaches along the South African coastline. Three gene regions were amplified and sequenced, namely the internal transcribed spacer region (ITS), a part of the nuclear large subunit (LSU), and part of the translation elongation factor 1 α (*tef1*) gene region. Molecular characteristics of strains isolated during this study showed high similarity with *Fusarium* isolates, which have previously been associated with high egg mortality rates in loggerhead sea turtles. This is the first record of *F. keratoplasticum*, *F. falciforme* and *F. crassum* isolated from stranded post-hatchling loggerhead sea turtles in South Africa.

The ascomycete genus *Fusarium* (*Hypocreales, Nectriaceae*) is widely distributed in nature and can be found in soil, plants and different organic substrates. This genus represents a diverse complex of over 60 phylogenetically distinct species. Some species, specifically those forming part of the *Fusarium solani* species complex (FSSC), are known pathogenic species, and have been associated with human, plant and animal infections—in both immunocompromised and healthy individuals. Phylogenetically, this group comprises three major clades, of which clade I forms the basal clade to the two sister clades II and III. Members of clade I and II are most often associated with plant infections and consequently have limited geographical distributions. Members of clade III represent the highest phylogenetic and ecological diversity and are most commonly associated with human and animal infections. Species represented in this clade are typically regarded as fast growing and produce large numbers of microconidia. This facilitates distribution within the host and its environment and promotes virulence. Clade III, further consists of three smaller clades, namely clades A, B and C. While clades A (also known as the *F. falciforme* clade) and C (also known as the *F. keratoplasticum* clade) consist predominantly of isolates from humans and animals, plant pathogens constitute most isolates represented in clade B.

*Fusarium* spp. have been identified in infections of marine animals including (but not limited to); bonnethead sharks (*Sphyrna tiburo*), scalloped hammerhead sharks (*Sphyna lewini*), and black spotted stingray (*Taeniura melanopsila*). Strains from this genus have been reported to cause skin and systemic infections in marine turtles, and are considered to be one of many threats to turtle populations worldwide causing egg infections and brood failure in 6 out of seven turtle species. Challenge inoculation experiments provided evidence of pathogenicity for *F. keratoplasticum*, a causative agent of sea turtle egg fusariosis (STEF) in loggerhead sea turtle populations in Cape Verde. Since then, *Fusarium* spp., or more specifically *F. falciforme* and *F. keratoplasticum* have increasingly been isolated from turtle eggs and nests. Subsequent research studies have isolated *F. falciforme* and *F. keratoplasticum* from infected eggs in turtle nests on beaches along the Atlantic, Pacific and Indian Oceans, as well as the Mediterranean and Caribbean Seas. Both *F. keratoplasticum* and *F. falciforme* are pathogenic to turtle eggs and embryos, and are able to survive independent of the hosts. In recent years, members from *F.
**Falciforme** and **F. keratoplasticum** of clade III, have been described as emerging animal pathogens, causing both localised and systemic infections\textsuperscript{6,16,17,23}. These infections can result in mortality rates as high as 80–90% in animal populations\textsuperscript{7,17}. Cafarchia and colleagues (2019) suggested that fusariosis should be included in differential diagnosis of shell and skin lesions in sea turtles and that species level identification is required to administer appropriate treatment and infection control\textsuperscript{12}.

Loggerhead sea turtles nest on the beaches of Southern Africa between November and January\textsuperscript{24,25}. Hatchlings that find their way into the ocean are carried south in the Aghulas current, with some turtles stranding on the South African coast, mainly between the months of March and May each year. Between 2015 and 2016, a total of 222 post-hatchling (turtles that have absorbed the yolk-sac and are feeding in open ocean but have yet to return to coastal waters to enter the juvenile stage) loggerhead sea turtles were admitted to a rehabilitation centre after stranding along the Indian and Atlantic Ocean coastline of South Africa, between Mossel Bay and False Bay (Fig. 1). During their time at the rehabilitation centre a number of these turtles developed skin lesions. Fungal dermatitis was diagnosed based on skin scrape cytology findings. Fungal strains resembling **Fusarium** were isolated from the affected areas.

The aim of this study was to characterise the strains isolated from skin lesions of post-hatchling loggerhead Sea turtles that washed up on beaches along the South African coastline, and to determine the molecular relationships between these isolates and those strains reported from literature that pose significant conservation risks to sea turtles from other geographic localities.

**Materials and methods**

**Gross observations and Fungal isolations.** Post-hatchling turtles with skin lesions were isolated from unaffected turtles. Clinical signs observed were as follows; excessive epidermal sloughing on the limbs, head and neck, where scales on the skin lifted easily and were frequently lost. A softening and sloughing of the carapace and plastron were observed, where scutes of the carapace and plastron became crumbly, soft and were frequently shed. Turtles were diagnosed with fungal skin infection if they had clinical signs of epidermal sloughing and a positive epidermal scrape. Epidermal scrapes taken from lesions of affected turtles were examined by light microscopy (20 to 50× objective) and deemed positive if significant numbers of hyphae were observed. For fungal isolation, samples (scrapings) were taken from affected areas of skin in a sterile manner and placed onto culture media. During 2015 and 2016, 10 fungal isolates were isolated from 10 clinically affected loggerhead sea turtles (**Caretta caretta**) onto marine phycomycetes isolation agar (12.0 g Agar, 1.0 g Glucose, 1.0 g Gelatin hydrolysate, 0.01 g Liver extract, 0.1 g Yeast extract, 1 000 mL Sea water) supplemented with streptomycin sul-
Phylogenetic analysis was performed using Maximum likelihood (ML) analysis, with GTR + I + G. The partitioning scheme and substitution models were selected using Partitionfinder v 2.1.1. Alignments were done in ClustalX using the L-INS-I option. The software package PAUP was used to construct the phylogenetic trees and confidence was calculated using bootstrap analysis of 1 000 replicates. A Bayesian analysis was run using MrBayes v. 3.2.6. The analysis included four parallel runs of 500 000 generations, with a sampling frequency of 200 generations. The posterior probability values were calculated after the initial 25% of trees were discarded.

**DNA extractions, molecular characterisation, and phylogenetic analyses.** Total genomic DNA was extracted from single spore colonies following incubation for 7 days on PDA. A heat lysis DNA extraction protocol was used. Extracted DNA were stored at −20 °C until needed. Molecular characterisation was performed based on 3 gene regions for 14 strains. The gene regions included internal transcribed spacer region (ITS), a part of the nuclear large subunit (LSU) and partial translation elongation factor 1-α (tef1) gene region. PCR reactions were performed in a total volume of 25 μL, containing 100–200 ng genomic DNA. Kapa ReadyMix (Kapa Biosystems; Catalog #KK1006) was used for PCR reactions. Conditions for the PCR amplification were as follows. Initial denaturation at 94 °C for 5 min, followed by 35 cycles at 94 °C for 45 s, 45 s annealing (see Table 1 for specific annealing temperatures) and 72 °C for 1 min, followed by a final extension at 72 °C for 7 min. Purified PCR products were sequenced by using BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) and an ABI PRISM 310 genetic analyser. Sequencing was done in one direction. Each sequence was edited in BioEdit sequence alignment editor v7.2.5. Phylogenetic analyses were conducted using the dataset from Sandoval-Denis et al. (2019) combining sequences of three loci (LSU, ITS and tef1) to identify species (Table 2 lists all the sequences included in the phylogenetic analyses). Alignments were done in ClustalX using the L-INS-I option. Phylogenetic analysis was performed using Maximum likelihood (ML) analysis, with GTR + I + G. The partitioning scheme and substitution models were selected using Partitionfinder v 2.1.1. The software package PAUP was used to construct the phylogenetic trees and confidence was calculated using bootstrap analysis of 1 000 replicates. *Geejayessia atrofusca* was used as an outgroup. A Bayesian analysis was run using MrBayes v. 3.2.6. The analysis included four parallel runs of 500 000 generations, with a sampling frequency of 200 generations. The posterior probability values were calculated after the initial 25% of trees were discarded.

**Morphological observation.** Agar plugs (6 mm diameter) of the selected isolates were transferred onto fresh PDA and Carnation leaf agar (CLA) plates and incubated at 26 ± 1 °C for 7 and 21 days, respectively for further morphological characterisation. Morphological characterisation was based on the taxonomic keys of Leslie and Summerell, 2006. Gross macro-morphology of all isolates was examined on PDA after 7 days, for further morphological characterisation. Agar plugs (6 mm diameter) of the chosen colonies 14 distinct isolates were identified for molecular characterisation. Agar plugs (6 mm diameter) of the chosen colonies were transferred onto PDA and incubated at 26 ± 1 °C for 7 days.

| Primer name | Primer sequence (5′–3′) | Annealing temperature (°C) | Reference |
|-------------|-------------------------|-----------------------------|-----------|
| ITS 1       | TCC GTA GGT GAA CCT GCG G | 51.1                        | 41        |
| ITS 4       | TCC TCG CTA TTA TAC GC   | 42                          |           |
| LSU-00021   | ATT ACC GCG TGA ACT TAA GC | 63.0                       | 42        |
| LSU-1170    | GCT ATC CGG AGA ATT GCG G | 65                          | 43        |
| E1           | ATG GGT AGG GAR GAC AAG AC | 53.6                       | 41        |
| E2           | GGA RGG ACC AGT SAT CAT GTT |                           |           |

Table 1. Primers used for amplification and sequencing.
| Species name | Strain number | Genbank accession number | Source | Origin | Reference |
|--------------|---------------|--------------------------|--------|--------|-----------|
| *Geejayessia atrofusca* (outgroup) | NRRL 22316 | AF178423 AF178392 AF178361 | *Staphylococcus* | USA | 28 |
| *F. ambrosium* | NRRL 20438 | AF178397 DQ236357 AF178332 | *Euwallacea fornicata* on *Camellia sinensis* | India | 28 |
| | NRRL 22346 = CBS 571.94T | EU329669 EU329669 FJ240350 | *Euwallacea fornicata* on *Camellia sinensis* | India | 28 |
| *F. bostrychoides* | CBS 130391 | EU329716 HM347127 | Human eye | Brazil | 28 |
| | CBS144.25NT | LR583704 LR583912 LR583597 | Soil | Honduras | 28 |
| | NRRL 31169 | DQ094396 DQ236438 DQ246923 | Human oral wound | USA | 28 |
| *F. ctenatum* | CBS 143229 T = NRRL54993 | KC808256 KC808256 KC808214 | *Stegostoma fasciatum* multiple tissues | USA | 28 |
| | NRRL 54992 | KC808255 KC808255 KC808213 | *Stegostoma fasciatum* multiple tissues | USA | 28 |
| *F. crassum* | CBS 144386 T | LR583709 LR583917 LR583604 | Unknown | France | 28 |
| | NRRL 46703 | EU329712 EU329712 HM347126 | Nematode egg | Spain | 28 |
| | NRRL 54722 = CBS 135854 T | JQ038014 JQ038014 JQ038007 | *Euwallacea fornicata* on *Persea americana* | Israel | 28 |
| | NRRL 62626 | KC691560 KC691560 KC691532 | *Euwallacea fornicata* on *Persea americana* | USA | 28 |
| *F. falciforme* | ML16006 | OM574602 ON237621 ON237635 | *Carettidae* post-hatching | South Africa | This study |
| | ML16011 | OM574607 ON237622 ON237636 | *Carettidae* post-hatching | South Africa | This study |
| | ML16012 | OM574608 ON237623 ON237637 | *Carettidae* post-hatching | South Africa | This study |
| | 033 FUS | KC573932 KC573883 | *Chelonia mydas* eggshells | Ecuador | 28 |
| | 078 FUS | KC573938 KC573884 | *Carettidae* *embryo* | Cape Verde | 7 |
| | 079 FUS | KC573939 KC573885 | *Carettidae* eggshells | Cape Verde | 7 |
| | 099 FUS | KC573956 KC573886 | *Carettidae* *embryo* | Cape Verde | 7 |
| | 142 FUS | KC573987 KC573887 | *Chelonia mydas* eggshells | Ecuador | 28 |
| | 181 FUS | KC573990 KC573888 | *Natator depressus* eggshells | Australia | 28 |
| | 182 FUS | KC573991 KC573889 | *Natator depressus* eggshells | Australia | 28 |
| | 209 FUS | KC574000 KC573890 | *Lepidochelys olivacea* eggshells | Ecuador | 28 |
| | 215 FUS | KC574002 KC573891 | *Lepidochelys olivacea* eggshells | Ecuador | 28 |
| | 219 FUS | KC574004 KC573892 | *Lepidochelys olivacea* eggshells | Ecuador | 28 |
| | CBS 121450 | JX435211 JX435211 JX435161 | Declined grape vine | Syria | 28 |
| | CBS 124627 | JX435184 JX435184 JX435134 | Human nail | France | 28 |
| | CBS 475.67 T | MG189935 MG189915 LT906669 | Human mycetoma | Puerto Rico | 28 |
| | ML16007 | OM574603 ON237617 ON237631 | *Carettidae* post-hatching | South Africa | This study |
| | ML16008 | OM574604 ON237618 ON237632 | *Carettidae* post-hatching | South Africa | This study |
| | ML16009 | OM574605 ON237619 ON237633 | *Carettidae* post-hatching | South Africa | This study |
| | NRRL 22781 | DQ094334 DQ236376 DQ246849 | Human cornea | Venezuela | 28 |
| | NRRL 28562 | DQ094376 DQ236418 DQ246903 | Human bone | USA | 28 |
| | NRRL 28563 | DQ094377 DQ236419 DQ246904 | Clinical isolate | USA | 28 |
| | NRRL 28565 | DQ094379 DQ236421 | Human wound | USA | 28 |
| | NRRL 31162 | DQ094392 DQ236434 | Human | Texas | 1 |
| | NRRL 32307 | DQ 094405 DQ236447 DQ246935 | Human sputum | Unknown | 28 |
| | NRRL 32313 | EU329678 EU329678 DQ246941 | Human corneal ulcer | Unknown | 28 |
| | NRRL 32331 | DQ094428 DQ236470 DQ246959 | Human leg wound | Unknown | 28 |
| | NRRL 32339 | DQ094436 DQ236478 DQ246967 | Human | Unknown | 28 |
| | NRRL 32540 | DQ094471 DQ236513 DQ247006 | Human eye | India | 28 |
| | NRRL 32544 | DQ094475 DQ23651 DQ247010 | Human eye | India | 28 |
| | NRRL 32547 | EU329680 EU329680 DQ247012 | Human eye | India | 28 |
| | NRRL 32714 | DQ094496 DQ236538 DQ247034 | Human eye | USA | 28 |
| | NRRL 32718 | DQ094500 DQ236542 DQ247038 | Human eye | USA | 28 |
| | NRRL 32729 | DQ094510 DQ236552 DQ247049 | Human eye | USA | 28 |
| | NRRL 32738 | DQ094519 DQ236561 DQ247058 | Human eye | USA | 28 |
| | NRRL 32754 | DQ094533 DQ236575 DQ247072 | Turtle nare lesion | USA | 28 |
| | NRRL 32778 | DQ094549 DQ236591 DQ247088 | Equine corneal ulcer | USA | 28 |
| | NRRL 32798 | DQ094567 DQ236609 DQ247107 | Human | USA | 28 |

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| Species name          | Strain number | Genbank accession number | Source          | Origin     | Reference |
|----------------------|---------------|--------------------------|-----------------|------------|-----------|
| **F. gamsii**         |               |                          |                 |            |           |
|                      | NRRL 43441    | DQ790522 DQ790522 DQ790478 | Human cornea    | USA        | 28        |
|                      | NRRL 43536    | EF453118 EF452966         | Human cornea    | USA        | 28        |
|                      | NRRL 43537    | DQ790550 DQ790550 DQ790506 | Human cornea    | USA        | 28        |
|                      | NRRL 52832    | GU170651 GU170651 GU170631 | Human toenail   | Italy      | 28        |
|                      | NRRL 54966    | KC808233 KC808233 KC808193 | Equine eye      | USA        | 28        |
|                      | NRRL 54983    | KC808248 KC808248 KC808206 | Equine eye      | USA        | 28        |
|                      | NRRL 43443    | DQ790529 DQ790529 DQ790485 | Human cornea    | USA        | 28        |
|                      | NRRL 43490    | EF453132 EF453132 EF452980 | Human eye       | USA        | 28        |
|                      | NRRL 46437    | GU170643 GU170643 GU170623 | Human toenail   | Italy      | 28        |
|                      | NRRL 46438    | GU170644 GU170644 GU170624 | Human toenail   | Italy      | 28        |
|                      | NRRL 46443    | GU170646 GU170646 GU170646 | Human foot      | Italy      | 45        |
|                      | NRRL 52704    | JF740908 JF740908 JF740786 | Tetranychus urticae | USA     | 28        |
| **F. keratoplasticum**|               |                          |                 |            |           |
|                      | CBS 143207 T  | DQ246462 DQ246462         | Human bronchoalveolar lavage fluid | USA | 28 |
|                      | NRRL 32794    | DQ236605 DQ247103          | Humidifier coolant | USA | 28 |
|                      | NRRL 43502    | DQ790532 DQ790532 DQ790488 | Human cornea    | USA        | 28        |
| **F. keratoplasticum (cont.)** |               |                          |                 |            |           |
|                      | 001 AFUS      | FR691753 IN939570         | Caretta caretta embryo | Cape Verde |            |
|                      | 001 CFUS      | FR691754 KC394706         | Caretta caretta embryo | Cape Verde |            |
|                      | 009 FUS       | FR691760 KC3973903        | Caretta caretta eggshells | Cape Verde | 7 |
|                      | 010 FUS       | FR691761 KC3973904        | Caretta caretta embryo | Cape Verde |            |
|                      | 013 FUS       | FR691764 KC3973907        | Caretta caretta eggshells | Cape Verde |            |
|                      | 014 FUS       | FR691757 KC3973908        | Caretta caretta eggshells | Cape Verde |            |
|                      | 015 FUS       | FR691759 KC3973909        | Caretta caretta eggshells | Cape Verde |            |
|                      | 016 FUS       | FR691758 KC3973910        | Caretta caretta eggshells | Cape Verde |            |
|                      | 018 FUS       | FR691765 KC3973911        | Caretta caretta eggshells | Cape Verde |            |
|                      | 019 FUS       | FR691766 KC3973912        | Caretta caretta eggshells | Cape Verde |            |
|                      | 021 FUS       | FR691768 KC3973913        | Caretta caretta embryo | Cape Verde |            |
|                      | 028 FUS       | KC573927 KC573914         | Chelonina mydas eggshells | Ecuador |            |
|                      | 029 FUS       | KC573928 KC573915         | Eretmochelys imbricata eggshells | Ecuador |            |
|                      | 030 FUS       | KC573929 KC573916         | Eretmochelys imbricata eggshells | Ecuador |            |
|                      | 034 FUS       | KC573933 KC573918         | Eretmochelys imbricata eggshells | Ecuador |            |
|                      | 036 FUS       | KC573935 KC573919         | Eretmochelys imbricata eggshells | Ecuador |            |
|                      | 223 FUS       | KC574007 KC573920         | Eretmochelys imbricata eggshells | Ascencion Island |            |
|                      | 230 FUS       | KC574010 KC573922         | Eretmochelys imbricata eggshells | Ascencion Island |            |
|                      | CBS 490.63 T  | LR583721 LR583929 LT906670 | Human           | Japan      | 28        |
|                      | FMR 7989 = NRRL 46696 | EU329705 EU329705 AM397219 | Human eye       | Brazil | 28 |
|                      | FMR 8482 = NRRL 46697 | EU329706 EU329706 AM397224 | Human tissue    | Qatar | 28 |
|                      | FRC-S 2477 T  | NR136900 JN235282 JN235712 | Indoor plumbing | USA | 28 |
|                      | ML16001       | OM574597 ON237611 ON237625 | Caretta caretta post-hatching | South Africa | This study |
|                      | ML16002       | OM574598 ON237612 ON237626 | Caretta caretta post-hatching | South Africa | This study |
|                      | ML16003       | OM574599 ON237613 ON237627 | Caretta caretta post-hatching | South Africa | This study |
|                      | ML16004       | OM574600 ON237614 ON237628 | Caretta caretta post-hatching | South Africa | This study |
|                      | ML16005       | OM574601 ON237615 ON237629 | Caretta caretta post-hatching | South Africa | This study |
|                      | ML16010       | OM574606 ON237620 ON237634 | Caretta caretta post-hatching | South Africa | This study |
|                      | ML16013       | OM574609 ON237623 ON237637 | Caretta caretta post-hatching | South Africa | This study |
|                      | ML16019       | OM574610 ON237624 ON237638 | Caretta caretta post hatching | South Africa | This study |
|                      | NRRL 22640    | DQ246842                  | Human cornea    | Argentina | 28 |
|                      | NRRL 22791    | DQ246853                  | Iguana tail     | Unknown   |           |
|                      | NRRL 28014    | DQ246872                  | Human           | USA        | 28        |
|                      | NRRL 28561    | DQ246902                  | Human wound     | USA        | 28        |
|                      | NRRL 32707    | DQ247027                  | Human eye       | USA        | 28        |
|                      | NRRL 32710    | DQ247030                  | Human eye       | USA        | 28        |
|                      | NRRL 32780    | DQ247090                  | Sea turtle      | USA        | 28        |
|                      | NRRL 32838    | DQ247144                  | Sea turtle      | USA        | 28        |
|                      | NRRL 32859    | DQ246718                  | Manatee skin    | USA        | 28        |
|                      | NRRL 43443    | EF453082 EF453082 EF453082 | Human           | Italy      | 28        |
|                      | NRRL 43490    | DQ790529 DQ790529 DQ790485 | Human eye       | USA        | 28        |
|                      | NRRL 43649    | EF453132 EF453132 EF452980 | Human eye       | USA        | 28        |
|                      | NRRL 46437    | GU170643 GU170643 GU170623 | Human toenail   | Italy      | 28        |
|                      | NRRL 46438    | GU170644 GU170644 GU170624 | Human toenail   | Italy      | 28        |
|                      | NRRL 46443    | GU170646 GU170646 GU170646 | Human foot      | Italy      | 45        |
|                      | NRRL 52704    | JF740908 JF740908 JF740786 | Tetranychus urticae | USA     | 28        |

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| Species name     | Strain number       | Genbank accession number       | Source                     | Origin   | Reference |
|------------------|---------------------|---------------------------------|----------------------------|----------|-----------|
| **F. lichenicola** | CBS 279.34 T        | LR583725 LR583933 LR583615       | Human                      | Somalia  | 28        |
|                  | CBS 483.96          | LR583728 LR583936 LR583618       | Air Brazil                 | Brazil   | 28        |
|                  | CBS 623.92 T        | LR583730 LR583938 LR583620       | Human necrotic wound       | Germany  | 28        |
|                  | NRRL 28030          | DQ094355 DQ226397 DQ246877       | Human                      | Thailand | 28        |
|                  | NRRL 34123          | DQ094645 DQ226687 DQ247192       | Human eye                  | India    | 28        |
| **F. metavorans** | CBS 135789 T        | LR583738 LR583946 LR583627       | Human pleural effusion     | Greece   | 28        |
|                  | NRRL 28018          | FJ240360 DQ246875                | Human                      | USA      | 28        |
|                  | NRRL 28019          | FJ240361 DQ246876                | Human                      | USA      | 28        |
| **F. parceramosum** | CBS 115695 T    | JX435199 JX435199 JX435149       | Soil                       | South Africa | 28        |
|                  | NRRL 31158          | DQ094389 DQ226431                | Human wound                | USA      | 28        |
| **F. petrophilum** | NRRL 32304          | DQ094402 DQ226444                | Human nail                 | USA      | 28        |
|                  | NRRL 32315          | DQ094412 DQ226454                | Human groin ulcer          | USA      | 28        |
|                  | NRRL 43812          | EF453205 EF453205 EF453054       | Contact lens solution      | Unknown  | 28        |
| **F. pseudensiforme** | CBS 241.93      | JX435198 JX435198 JX435148       | Human mycetoma             | Suriname | 28        |
|                  | FRC-S 1834 = CBS 125729 T | JX435199 JX435199 JX435149 | Human mycetoma | Suriname | 28        |
| **F. pseudotonkinense** | CBS 112101 | LR583772 LR583977 LR583653       | Human vocal prosthesis     | Belgium  | 28        |
|                  | NRRL 124893         | JX435191 JX435191 JX435141      | Human nail                 | France   | 28        |
|                  | GJS 09-1466 T       | KT313633 KT313633 KT313611       | Solanum tuberosum          | Slovenia | 28        |
|                  | NRRL 31168          | DQ094333 DQ226375 DQ246848      | Human toenail              | New Zealand | 28        |
|                  | NRRL 32492          | DQ094395 DQ226437 DQ246922      | Human toe                  | USA      | 28        |
|                  | NRRL 32373          | EU329679 EU329679 EU329690      | Human                      | USA      | 28        |
|                  | NRRL 32791          | DQ094518 DQ226560 DQ247057      | Human eye                  | USA      | 28        |
|                  | NRRL 32810          | DQ094557 DQ226619 DQ247118      | Human corneal ulcer        | USA      | 28        |
|                  | NRRL 43468          | EF453093 EF453093 EF453094      | Human eye                  | USA      | 28        |
|                  | NRRL 43474          | EF453097 EF453097 EF453095      | Human eye                  | USA      | 28        |
|                  | NRRL 44896          | GU170639 GU170639 GU170619      | Human toenail              | Italy    | 28        |
|                  | NRRL 46598          | GU170648 GU170648 GU170628      | Human toenail              | Italy    | 28        |
| **N. solani**    | CBS 142481 T        | LR583779 LR583984 LR583658       | Compost yard debris        | Belgium  | 28        |
|                  | CBS 144388          | LR583780 LR583985 LR583659       | Greenhouse humic soil      | Belgium  | 28        |
|                  | CBS 260.54          | LR583776 LR583981 LR583657       | Unknown                    | Unknown  | 28        |
|                  | NRRL 22239          | LR583777 LR583982 DQ247562      | Nematode egg               | Germany  | 28        |
| **F. stericola** | CBS 124892          | JX435189 JX435189 JX435139      | Human nail                 | Gabon    | 28        |
|                  | CBS 143214 T        | DQ094617 DQ226659 DQ247163      | Human wound                | USA      | 28        |
|                  | NRRL 28000          | DQ094348 DQ226390 DQ246866      | Human                      | USA      | 28        |
|                  | NRRL 32316          | DQ094413 DQ226455 DQ246944      | Human cornea               | USA      | 28        |
|                  | NRRL 54972          | MG189940 MG189925 KC808197      | Equine eye                 | USA      | 28        |
| **F. tonkinense** | CBS 115.40 T        | MG189941 MG189926 LT906672      | Musa sapientum             | Vietnam  | 28        |
|                  | CBS 222.49          | LR583783 LR583988 LR583661      | Euphorbia fulgens          | Netherlands | 28        |
|                  | NRRL 43811          | EF453204 EF453204 EF453053      | Human cornea               | USA      | 28        |
| **F. vasinfecta** | CBS 101957          | LR583797 LR584002 LR583676      | Human blood, sputum and wound | Germany  | 28        |
|                  | CBS 446.93 T        | LR583791 LR583966 LR583670      | Soil                       | Japan    | 28        |
|                  | NRRL 43467          | EF453092 EF453092 EF453090      | Human eye                  | USA      | 28        |
| **Fusarium sp. (AF1)** | NRRL 22231      | KC691570 KC691570 KC691542      | Beetle on Hevea brasilensis | Malaysia | 28        |
|                  | NRRL 46518          | KC691571 KC691571 KC691543      | Beetle on Hevea brasilensis | Malaysia | 28        |
|                  | NRRL 46519          | KC691572 KC691572 KC691544      | Beetle on Hevea brasilensis | Malaysia | 28        |
| **Fusarium sp. (AF6)** | NRRL 62590      | KC691574 KC691574 KC691546      | Euwallacea fornicatus on Persea americana | USA | 28        |
|                  | NRRL 62591          | KC691573 KC691573 KC691545      | Euwallacea fornicatus on Persea americana | USA | 28        |

Continued
| Species name               | Strain number | Genbank accession number | ITS       | LSU       | EF         | Source                        | Origin          | Reference |
|---------------------------|---------------|--------------------------|-----------|-----------|------------|-------------------------------|-----------------|-----------|
| Fusarium sp. (AF7)        | NRRL 62610    | KC691575                 | KC691575 | KC691547  | F. euwallacea sp. on Persea americana | Australia       | 28        |
|                           | NRRL 62611    | KC691576                 | KC691576 | KC691548  | F. euwallacea sp. on Persea americana | Australia       | 28        |
| Fusarium sp. (AF8)        | NRRL 62585    | KC691582                 | KC691582 | KC691554  | F. euwallacea fornicatus on Persea americana | USA             | 28        |
|                           | NRRL 62584    | KC691577                 | KC691577 | KC691549  | F. euwallacea fornicatus on Persea americana | USA             | 28        |
| Fusarium sp. (FSSC 12)    | NRRL 23642    | DQ094329                 | DQ23671   | DQ246444  | Penicillus japonicus gill | Japan           | 28        |
|                           | NRRL 25392    | EU329672                 | EU329672  | DQ246461  | American lobster | USA             | 28        |
|                           | NRRL 32309    | DQ094407                 | DQ236449  | DQ246937  | Sea turtle | USA             | 28        |
|                           | NRRL 32317    | DQ094414                 | DQ236456  | DQ246945  | Treefish   | USA             | 28        |
|                           | NRRL 32821    | DQ094587                 | DQ236629  | DQ247128  | Turtle egg | USA             | 28        |

Table 2. *Fusarium* strains included in the phylogenetic analyses.

Results

Molecular characterisation and Phylogenetic analyses. Phylogenetic analyses (Figs. 2 and 3) showed 3 (*F. falciforme*, *F. keratoplasticum* and *F. crassum*) distinct species. A phylogenetic tree generated from the combined dataset of LSU, ITS and *tef1* gene regions, represented 3 lineages within the *Fusarium solani* species complex (FSSC). The maximum likelihood (ML) analysis included 135 taxa (including the outgroup). In the analyses, 14 strains isolated during this study, aligned with three species within *Fusarium*. Seven strains (ML16001; ML16013; ML16005; ML16004; ML16003; ML16002; ML16010) grouped with the *F. keratoplasticum* clade with a strong bootstrap support. Four strains (ML16007; ML16008; ML16009; ML16019) grouped within the more diverse *F. falciforme* clade. Another three strains (ML16006; ML16011; ML16012) grouped with *F. crassum*. Secondary phylogenetic analysis of the ITS and LSU gene regions, included 118 taxa (including the outgroup). These analyses confirmed the findings of primary phylogenetic analyses and showed that isolates from this study aligned with isolates that were previously associated with turtles and turtle eggs.

Morphological observation. Three strains expressed significant different morphological characteristics compared to other strains isolated during this study. These three strains were relatively fast growing on PDA, reaching a colony size of 70–75 mm diameter after 7 days of incubation at 26 ± 1 °C. White, flat floccose mycelium with light peach to yellow centre. White to pale light yellow on the reverse side. On CLA, incubated at 26 ± 1 °C, reaching a colony size of 80–90 mm diameter in 7 days. Microconidia were oval, ellipsoidal to sub-cylindrical in shape, with 0–1 septum, smooth and thin walled arranged in false heads at the tip of long monophialides. Average aseptate microconidia measured as follows for the three strains (n = 30 per strain); 11.5 µm (± 1.25) × 4.00 µm (± 0.5), 12.0 µm (± 1.0) × 4.0 µm (± 0.5) and 11.5 µm (± 2.0) × 4.25 µm (± 0.4). Microconidia with one septa measured as follows (n = 30 per strain); 15.0 µm (± 2.0) × 4.25 µm (± 0.5), 15.0 µm (± 1.5) × 4.0 µm (± 0.5) and 15.5 µm (± 5.0) × 4.5 µm (± 0.5). Microconidia were fusiform in shape with the dorsal sides more curved than the ventral sides, blunt apical cells and barely notched foot cells. Microconidia consisted of 3–4 phialides. Average aseptate microconidia measured as follows for the three strains (n = 30 per strain); 11.5 µm (± 0.5) and 30.0 µm (± 1.0) × 5.0 µm (± 0.5). Sporodochia ranged from clear to beige in colour. Chlamydospores were first observed after 14 days of incubation on CLA plates, and were globose in shape with rough walls, positioned terminally, sometimes single but mostly in pairs. Distinct hyphal coils were observed in all three strains (Fig. 4). The morphology is consistent with that described for *N. crassum* (Fig. 4).

Discussion

*Fusarium* infections, specifically *F. keratoplasticum* and *F. falciforme* have been reported from infected eggs and embryos of turtle species, including endangered species, at major nesting sites along the Atlantic, Pacific and Indian Oceans, as well as the Mediterranean and Caribbean Sea. Management strategies to mitigate emerging fungal diseases, like *Fusarium* infections in turtle eggs, are influenced by identifying whether a pathogen is novel or endemic and the understanding of its ecology and distribution. A novel pathogen gains access to and infects naïve hosts as a result of migration of the pathogen or the development of novel pathogenic genotypes, in contrast endemic pathogens occur naturally in the host’s environment, but shifts in environmental conditions and/or host susceptibility influence pathogenicity. Thus, effective management strategies to mitigate novel pathogens should aim at preventing pathogen introduction and expansion, while disease caused by endemic pathogens relies on an understanding of environmental and host factors that influence disease emergence and severity. Phylogenetic analysis provides important information to assist in understanding the ecology, introduction and distribution of infectious agents. The first aim of this study was to use multigene phylogenetic analyses to identify *Fusarium* strains isolated from the carapace, flippers, head, and neck area of post-hatchling loggerhead sea turtles (*Caretta caretta*) with fungal skin infections that stranded along the South African coastline and kept at a rehabilitation centre. The genus *Fusarium* was recently revised, with an attempt to standardise the taxonomy and nomenclature after a lack of formal species descriptions, Latin names and nomenclatural type...
Figure 2. Maximum likelihood analysis of *Fusarium* species isolates based on three loci, translation elongation factor 1 α (*tef1*), large subunit (LSU) and internal transcribed standard (ITS). Numbers within the tree represent the bootstrap values of 1 000 replicates, followed by the posterior probability (italics). Strains isolated during this study are marked with a red asterisk (*).
Figure 3. Maximum likelihood analysis of *Fusarium* species isolates from other marine animals based on two loci, large subunit (LSU) and internal transcribed standard (ITS). Numbers within the tree represent the bootstrap values of 1 000 replicates, followed by the posterior probability (italics). Strains isolated during this study are marked with a red asterisk (*).
specimens were identified. Strains from this study grouped with three Fusarium species of which two species, *F. keratoplasticum* and *F. falciforme*, were previously reported to occur on animal hosts, including turtles. The third species, *F. crassum* is rather surprising as this species is only known from a human toenail and nematode eggs, while the origin of the type strain is unknown. Three strains (ML16011, ML16012 and ML16006) grouped with two *F. crassum* strains. Strain identifications were confirmed with the morphological characteristics that agreed with species descriptions published in 2019, with the one exception of chlamydospore wall texture for *F. crassum*. Chlamydospore walls in this study for all three *F. crassum* strains were smooth, while previously it has been documented with a rough texture.

Turtle egg fusariosis (STEF) is a disease that has increasingly been reported over the last decade and is considered a potential conservation threat to six out of seven species of marine turtles. Skin disease and systemic infections caused by *Fusarium* species has been reported in adult and subadult turtles and in captive reared hatchlings, but has not been reported in post-hatching loggerhead sea turtles (*C. caretta*) undergoing rehabilitation. Clinical signs reported in juvenile, subadult and adult loggerhead sea turtles (*C. caretta*) with *Fusarium* infections were localised and generalised lesions of the skin and carapace, consisting of areas of discoloration and loss of shell. Clinical signs observed in post-hatching loggerhead sea turtles (*C. caretta*) in this study were similar, but generalised sloughing of scales on the limbs and head, and a soft, crumbly carapace and plastron were more common than focal lesions. Histopathology was not performed in this study to confirm the association of fungal hyphae with pathological changes in the skin, and, therefore, the role of the *Fusarium* isolates in the skin lesions cannot definitively be identified (as isolation of fungus could be from normal skin flora or the environment), however, fungal hyphae, often in dense mats, were seen in epidermal scrapes from affected turtles (Online Resource 2). Although *Fusarium* isolates (and other fungi) have been identified in the skin of healthy adult *C. caretta*, a finding of numerous hyphae (hyphal mats) in skin scrapings would not be considered a normal finding in healthy turtle skin and thus it is considered likely that the fungal elements observed, and therefore the isolates identified, were associated with the observed pathology. The epidemiology of turtle pathogenic isolates *F. keratoplasticum* and *F. falciforme* in sea turtle nesting sites are not fully understood, however, it has been suggested that tank substrates and/or biofilms forming in the water supply infrastructure or filtering systems may act as a source of infection, to traumatised and immunocompromised sea turtles.

Investigations into the source of infection were not undertaken in this study, so it is not clear if the fungal isolates originated in the rehabilitation environment or were present in the skin on admission. Cafarchia and colleagues (2019) found increased length of stay to be a risk factor for fungal colonisation, where turtles staying in a rehabilitation centre for over 20 days were more frequently colonised with *Fusarium*. Loggerhead sea turtles (*C. caretta*) in this study exhibited clinical signs around 20–30 days after admission and it is likely that most individuals experienced some degree of immunocompromise in the initial stages of rehabilitation. This, combined with physical skin trauma that may be present on admission may have provided a suitable environment for fungal colonisation. The second aim of study was to establish the phylogenetic relationship between *F. keratoplasticum* and *F. falciforme* strains isolated during this study and strains that were previously associated
with brood failure and high mortality rates\textsuperscript{17,18}. Combined sequence data of the ITS and LSU regions revealed that seven of the strains formed part of the monophyletic \textit{F. keratoplasticum} clade. Strains isolated during this study showed a close phylogenetic relation with other species in this clade, consisting of species that were previously isolated from Hawksbill (\textit{E. imbricata}) and green sea turtle (\textit{C. mydas}) eggs shells from nesting beaches along the Pacific Ocean in Ecuador\textsuperscript{15,16}. Furthermore, phylogenetic analyses of the \textit{F. falciforme} group showed close resemblance to strains that were previously isolated from olive ridley sea turtle (\textit{L. olivacea}), green sea turtle (\textit{C. mydas}), flatback sea turtle (\textit{N. depressus}) and loggerhead sea turtle (\textit{C. caretta}) egg shells and \textit{C. caretta} embryos on nesting beaches in Australia, Cape Verde and Ecuador, Turkey, along the Pacific, Atlantic and Indian Ocean\textsuperscript{15,17-19,21}. In addition, these strains showed a close resemblance to a strain that was previously isolated from a lesion in an adult turtle nare from the USA\textsuperscript{29}. Based on the ITS and LSU gene regions, a genetic relationship exists between \textit{Fusarium} species associated with turtle egg infections (also known as STEF) and \textit{Fusarium} species isolated from post-hatching loggerhead sea turtles (\textit{C. caretta}) that stranded on beaches in South Africa along the Indian ocean.

Infections caused by members of this genus have been reported in numerous other aquatic animals in the past\textsuperscript{6,9,20}, but for many of these, identification has been limited and mostly based on morphological characteristics. Many reports based on morphology only identified causative agents as \textit{Fusarium} (\textit{F. solani}), lacking further identification. Accurate identification of pathogenic \textit{Fusarium} members is essential for epidemiological purposes and for assisting in management programs, however, more research is required to complete the puzzle and fully understand the ecology and distribution of these pathogens, especially amongst reptiles and aquatic animals. This is the first confirmed record of \textit{F. keratoplasticum} and \textit{F. falciforme} strains isolated from post-hatching loggerhead sea turtles (\textit{C. caretta}) from the South African coastline that were not associated with nesting sites. This is also the first record of \textit{F. crassum} to be associated with loggerhead sea turtles.

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**Author contributions**

MR.G.-L. – First Author, conducted all laboratory work and wrote manuscript. K.J. – Responsible for phylogeny and assisted in writing of the manuscript. All authors reviewed the manuscript.

**Competing interests**

The authors declare no competing interests.

**Additional information**

**Supplementary Information** The online version contains supplementary material available at [https://doi.org/10.1038/s41598-022-06840-1](https://doi.org/10.1038/s41598-022-06840-1).

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