Is fish food from aquarium suppliers a vector for non-native zooplankton to New Zealand?

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Abstract

There have been a number of recent records of non-native zooplankton invasions in New Zealand, but the transport vectors leading to their introduction are not well understood. Commercially available fish foods containing zooplankton are widely used in home aquariums and the aquaculture industry. These foods are either imported to, or cultured within, New Zealand, and sold as freeze-dried, sun-dried or frozen adults, as capsulated or decapsulated cysts, or as live individuals. In this study, fish food containing *Daphnia* O.F. Müller, 1785 (Cladocera), *Artemia* (brine shrimp) and *Brachionus* (rotifers) were bought from a variety of commercial suppliers within the North Island, New Zealand, which were cultured and manufactured domestically or internationally. Live *Daphnia* samples were identified morphologically and were found to contain a variety of both native and non-native *Daphnia* species, as well as copepods, ostracods, rotifers and other non-*Daphnia* cladoceran species. Freeze-dried and sun-dried diapausing eggs of *Daphnia* and the rotifer *Brachionus* were incubated in artificial pond water, Aachener Daphnien Medium (ADaM) and filtered pond water, but hatching of these were unsuccessful. In contrast, *Artemia* bought as both capsulated and decapsulated cysts were successfully hatched in water with a salinity of 25 ppt and temperatures of 20 °C. The resulting *Artemia* populations were either parthenogenic or sexual, varying by manufacturer. Frozen whole adult *Artemia* were found to carry eggs but could not be hatched. Due to the risk of misidentifications from morphological identification, a 658-bp fragment from the 5′ region of the COI gene was amplified from tissue samples. The phylogenetic tree constructed from the COI gene sequences identified distinct clades corresponding to four species: *A. franciscana*, *A. parthenogenetica* (both hatched from eggs as viable populations), *A. sinica*, and *A. salina* (both identified in food, but not viable). The

BLAST analysis indicated that the capsulated cysts from USA and German manufacturers contained *Artemia franciscana*, while the Chinese supply contained *A. parthenogenetica*. Decapsulated cysts from a Chinese manufacturer hatched into *A. parthenogenetica*. While *A. franciscana* can be legally imported into New Zealand, *A. parthenogenetica* is not listed in the Import Health Standard for 'Fish food and fish bait' under the Biosecurity Act 1993. The morphological identifications matched the BLAST analysis data for *Daphnia.* Non-native *Daphnia pulex* was recorded from live samples from Te Aroha, Hamilton and Wellington (LID5). An Auckland sample and another Wellington sample comprised of native *D. carinata* s.l. Overall, commercially available fish food from aquarium suppliers can act as a vector for non-native zooplankton to New Zealand.

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Introduction

1.1 Food for aquarium fish

The origins of “fish keeping”, the world's second most popular hobby next to photography (Tekade, 2023), can be traced back centuries, with roots in Asia and Egypt (Asche et al., 2008). This practice gained widespread popularity in North America, Britain, and Germany during the mid-19th century (Saint-Erne, 2024). Technological advancements have significantly influenced the ornamental fish trade and fish-keeping hobby (Asche et al., 2008). For example, advances in transportation, including the transition from cargo ships to airline freight, along with the adoption of plastic bags and Styrofoam containers, significantly enhanced the ability to transport ornamental fish (Duggan, 2011; Novák et al., 2020). Additionally, the widespread availability of electricity facilitated essential aquarium technologies such as heating, artificial lighting, aeration, and filtration, while the development of artificial seawater and protein skimmers contributed to the growth of ornamental marine fish-keeping (Duggan, 2011). As such, the keeping of ornamental fish is an expanding, globally popular hobby (Rixon et al., 2005; Shepherd, 2008; Novák et al., 2020; Vasantharajan, 2023). It is estimated that over one billion freshwater fish, representing more than 5,300 species, are traded internationally as pets each year (Maceda-Veiga et al., 2014) and the value of the global aquarium trade is US $15 – 30 per annum (Evers et al., 2019).

Unlike marine fish, most freshwater ornamental fish and invertebrates are raised in captivity (Tlusty, 2002). In captivity, high survival rates, high growth rates, bright and attractive body colourations and high fecundity are some important features of fish quality, which can be

gained via good nutrition (Joshi et al., 2021; Yadav & Semwal, 2021). The desired nutritional requirements for the well-being of fish can be fulfilled by feeding them high-quality food (Manam, 2023). Ideally, fish food should be balanced, with proteins, lipids, carbohydrates, minerals and vitamins provided in adequate amounts (Velasco-Santamaría & Corredor, 2011). These nutrients play a vital role in maintaining the overall health of the fish (Madkour et al., 2023).

Nutrient requirements differ based on species, sex, habitat, and overall health condition (Manam, 2023). Furthermore, the nutritional requirements for each life stage of fish are different. For example, the protein requirement as a proportion of the diet of juvenile goldfish (29%) is lower than the protein requirement of the larvae (53%) (Velasco-Santamaría & Corredor, 2011), and compared to other life stages, juvenile fish require a diet rich in protein but low in energy, whereas mature adult fish need a diet higher in energy and lower in protein (Manam, 2023). Importantly, newly hatched larvae do not possess a well-developed digestive system with all the necessary digestive enzymes, and face difficulties in the digestion of formulated fish food. Therefore, newly hatched larvae rely on live prey (Harpaz et al., 2005). When fish are foraging in their natural habitats, they obtain a balanced diet from naturally available food sources (Velasco-Santamaría & Corredor, 2011). However, when kept in aquariums, aquarists must provide a properly balanced diet with an easily digestible form of food that is well-fitted with the fish's life-history stages (Saikia, 2023).

Different species of fish have different food preferences according to whether they are herbivorous, carnivorous or omnivorous (Velasco-Santamaría & Corredor, 2011). Many carnivorous aquarium fish species prefer rotifers, protozoans, small crustaceans such as copepods and cladocerans, and insect larvae (Suresh, 2003). Carnivorous fish may prefer live food as they can hunt their prey (i.e., *Artemia* Leach, 1819) (Suresh, 2003). This may trigger

the food-capturing behaviour and encourage more hunting, which enhances fish growth (Aiswarya et al., 2024).

Fish food is available as either live food or processed products. Processed fish foods are commonly available in the form of flakes and pellets made up of ingredients such as fish meal, ground rice, dried yeast, oatmeal, algae meal, and other items. Higher quality feeds include freeze-dried, sun-dried or frozen algae or invertebrates, or as capsulated or decapsulated *Artemia* eggs (García et al., 2011; Yadav & Semwal, 2021). Other than the capsulated eggs, all of these forms are ready to feed to fish without further processing (Laviña & Figueroa, 1978; Joshi et al., 2021; Madkour et al., 2023). Freeze-dried and sun-dried feed is usually available in sealed plastic containers in the form of flakes, pellets, granules or crushable cubes. Floatable flakes are more suitable for smaller fish while sinking and floating pellets are for larger fish (Yadav & Semwal, 2021). Freeze-dried and frozen foods are commonly made up of whole tubifex worms, blood worms, daphnids, cyclopoid copepods, rotifers, mosquito larvae and *Artemia*, and are mainly preferred by tropical and marine fish that demand special nutritional requirements (Yadav & Semwal, 2021). Live fish feed comprises cladocerans, bloodworms, copepods, rotifers, earthworms, and *Artemia* larvae (Yadav & Semwal, 2021). Capsulated eggs are hatched to develop live *Artemia* nauplii to supply live feed to the early stages of fish (Suresh, 2003).

1.2 Live feeds

According to Lim et al. (2001), the best production of fish larvae, fry and fingerlings depends on the availability of live food. Early stages of fish larvae do not feed on formulated forms of fish feed such as mash, wet balls, flakes, crumbles, and pellets (Manam, 2023). In this stage, they only feed on live organisms and feeding *Artemia* nauplii is the best-known practised

solution (Suresh, 2003). Other than *Artemia*, macro- and micro-zooplankton such as daphnids, copepods and rotifers are used by aquaculturists as an alternative live feed, but have not been as successful as *Artemia* for growth of fish (Tamaru et al., 1997; Lim et al., 2001; Chong et al., 2002; Lian Chuan et al., 2003; Lim et al., 2003). However, for very early larval fish stages, rotifers, especially those belonging to the genus *Brachionus*, are commonly used due to their slow mobility, small body size and high nutritional value (Lim et al., 2003). In New Zealand, *Artemia* and *Daphnia* are the most commonly used live foods for aquarium fish (personal observation).

The life history of *Artemia* is composed of 15 moults, with individuals reaching maturity from around 20 days of hatching (Gajardo et al., 2002; Madkour et al., 2023). Adult female *Artemia* are capable of producing around 250 embryos per brood and up to 20 broods during the entire lifespan (Abatzopoulos et al., 2002). They can reproduce either ovoviviparously, with eggs hatched within the body, or oviparously, with eggs hatched outside the body (Abatzopoulos et al., 2002). Ovoviviparity is observed under favourable environmental conditions, whereas oviparity is exhibited when the environmental conditions become unfavourable (e.g., through high salinities and temperatures, low oxygen concentrations, food scarcity, and short daylight conditions (Abatzopoulos et al., 2003). A key advantage of using *Artemia* as a live food is that they can be produced from cysts, which can be dried and stored for years (Suresh, 2003). These cysts remain metabolically inactive and do not develop further, as long as they are kept in dry conditions. On the other hand, a short period of hydration (around 24 hours) induces the development of the embryo-producing *Artemia* nauplii (Suresh, 2003). With their high fecundity and high nutritional value, *Artemia* larvae have become popular among aquaculturists as a food source for fish and shellfish larvae. *Artemia* larvae are capable of supplying not only the vital nutrients but also the crucial enzymes for the early stages of fish and shellfish larvae (Sorgeloos et al., 2001). Furthermore,

live adult *Artemia* is an ideal feed for some fish species (Lian Chuan et al., 2003), including coho salmon (*Oncorhynchus kisutch*) in aquaculture (Jihye et al., 1996).

1.3 Diversity of *Daphnia* in New Zealand

Daphnia is a cosmopolitan cladoceran genus with a high diversity (Burns et al., 2017). However, the diversity of *Daphnia* in New Zealand is somewhat low relative to elsewhere. According to Chapman & Lewis (1976), until the 1950s there were only two *Daphnia* species known from New Zealand; the native *Daphnia thomsoni* Sars, 1894 (then referred to as *D. carinata*; see Burns et al., 2017) and non-native *D. obtusa* Kurz, 1874. *Daphnia obtusa* represents a species complex, and one of the lineages was found in a small pond containing non-native macrophytes, indicating a possible recent introduction via these plants (Burns et al., 2017). Two *Daphnia* species have invaded relatively recently, a North American *Daphnia pulex*, comprised of a lineage or lineages of the *D. pulex* complex, and *D. galeata*, which have rapidly spread throughout New Zealand (Duggan et al., 2006; Duggan et al., 2012). According to molecular analyses carried out by Ye et al. (2021), the lineages of the *Daphnia 'pulex'* complex from the North Island and the South Island are different. Their analysis indicated that in the South Island, the invasive *Daphnia* species was *Daphnia pulicaria* Forbes, 1983, whereas in the North Island, the invasive populations were hybrids of *D. pulicaria* and *D.* cf. *pulex, sensu* Hebert,1978 (Ye et al., 2021). Finally, *Daphnia tewaipounamu* was described in 2017, being found from some subalpine ponds in the South Island, and is considered to be an endemic species within the *D. carinata* species complex (Burns et al., 2017). Therefore, New Zealand *Daphnia* diversity comprises two endemic species and four introduced species (Burns et al., 2017; Ye et al., 2021).

1.4 Current status of *Artemia* diversity globally and in New Zealand

The first record of *Artemia* with scientific drawings was made by Schlösserin in 1756 from Lymington, England (Asem, 2008). Later, in 1758, Linnaeus described this organism as *Cancer salinus*, while in 1819, Leach renamed the species *Artemia salina* Linnaeus, 1758 (Asem et al., 2010). *Artemia*, commonly known as brine shrimp, is a crustacean (Crustacea, Branchiopoda, Anostraca) that prefers extremely halophilic conditions and shows a cosmopolitan distribution, occurring on every continent except Antarctica (Triantaphyllidis et al., 1998; Abatzopoulos et al., 2002; Muñoz & Pacios, 2010). *Artemia* inhabits saline and hypersaline (10-340 Practical Salinity Units, PSU) environments found in coastal areas and inland, such as salt lakes, wetlands, lagoons and salt ponds (Gajardo et al., 2002; Abatzopoulos et al., 2003; Muñoz & Pacios, 2010; Veeramani et al., 2019). The extreme tolerance capability of variability in oxygen concentrations, salinities, different ionic compositions (sulphate, chloride, or carbonate-rich water), desiccation, high UV light conditions, temperatures and highly adaptable physiology, have allowed *Artemia* to have its widespread distribution (Varó et al., 2015; Camara, 2020; Madkour et al., 2023). According to Abatzopoulos et al. (2002), *Artemia* populations have been recorded from more than 600 sites worldwide. Recent findings by Asem et al. (2023) indicate that *Artemia* comprises nine species of sexually reproducing species, together with obligate parthenogenetic lineages. Of the nine bisexual species, three can be found in the New World while the others are from the Old World. The New World native species are *Artemia franciscana* Kellogg, 1906 (North, Central and South America), *A. persimilis* Piccinelli and Prosdocimi, 1968 (Argentina and Chile), and *A. monica* Verrill, 1869 (Mono Lake, USA). The Old World native species are *A. salina* Linnaeus, 1758 (Europe and Africa), *A. urmiana* Günther, 1899 (Urmia lake, Iran, and the Crimean Peninsula), *A. sinica* Cai, 1989 (Central and

East China), *A. tibetiana* Abatzopoulos, Zhang and Sorgeloos, 1998, *A. sorgelossi* Asem, Eimanifar, Hontoria, Rogers and Gajardo, 2023 (Tibetan Plateau and China) and *A. amati* Asem, Eimanifar, Hontoria, Rogers & Gajardo, 2023 (Kazakhstan) (Asem et al., 2024). *Artemia* genetic diversity is structured into locally adapted sexual populations, influenced by the ecological variability of lakes and lagoons and their island-like, allopatric (regionally endemic) distribution (Asem et al., 2023; Asem et al., 2024).

According to Asem et al. (2024), the first documented case of parthenogenetic *Artemia* was reported by von Siebold in 1871. Later studies revealed that parthenogenetic lineages are obligate (Browne & Hoopes, 1990). These obligate parthenogenetic lineages have originated in the Old World and Australia through hybridization followed by backcrosses between asexual and sexual relatives of various Asian sexual species (Rode et al., 2022). The parthenogenetic lineages show polyploidy, such as diploidy, triploidy, tetraploidy, pentaploidy, and nonaploidy (Zheng & Sun, 2013). Since these parthenogenetic lineages have different ancestries, they form a polyphyletic group. Di- and tri-polyploid lineages have been found to exhibit an evolutionary relatedness to *A. urmiana* while tetra- and penta- lineages are related to *A. sinica* (Abatzopoulos et al., 2002).

There are no *Artemia* species native to New Zealand (Chapman & Lewis, 1976). However, *Artemia* has established a non-native population in New Zealand, likely due to anthropogenic activities such as aquaculture (Haslett & Wear, 1985). According to Knight (1974), Lake Grassmere / Kapara Te Hau, located on the east coast of the South Island, is used for solar salt extraction; they found this lake to contain what they believed to be *Artemia salina*. The salt extraction plant was established in 1954, and at that time Lake Grassmere was thought not to be a habitat for *Artemia*. In 1974, brine shrimps were recorded from this lake and by this time brine shrimp eggs were being imported to New Zealand from Great Salt Lake, Utah, USA (Knight 1974). As such, *Artemia salina* was considered to not be acceptable as the

taxonomic name for the species present in Lake Grassmere (Bowen & Sterling, 1978) and the *Artemia* species in Lake Grassmere has since been referred to as *A. franciscana*, consistent with a North American origin (Haslett & Wear, 1985; Robert & Stephen, 1986). According to the New Zealand Inventory of Biodiversity, *A. franciscana* is the only *Artemia* species with an established population in New Zealand (Gordon, 2010). Nevertheless, the Biosecurity Act (1993) via the Import Health Standards for Fish Food and Fish Bait and for Personal Consignments of Animal Products for *Artemia* states that both *A. fransicana* and *A. salina* can be imported; the allowance of both species being importable into New Zealand may be based on both of these names being used in the New Zealand scientific literature prior to 29 July 1998, as outlined by the Hazardous Substances and New Organisms Act (HSNO) 1996 (Ministry for Primary Industries, 2023; Ministry for Primary Industries, 2025).

1.5 Dispersal methods of *Artemia*

Artemia can be dispersed over long distances either by natural methods such as migratory birds and water currents or by anthropogenic activities, such as through the ornamental fish trade or aquaculture (Abatzopoulos et al., 2002). Human-aided introduction of *Artemia* into a new location may be due to intentional or unintentional actions. The first deliberate introduction of *Artemia* beyond its native range occurred in the late 1970s into salt ponds in Macau (Rio Grande do Norte), Brazil. Since then, further introductions have been carried out to facilitate *Artemia* cyst production (e.g., introductions took place in the 1970s in the Pacific Islands and Brazil) (Camara, 2020).

Using cryptobiotic eggs in the aquaculture industry and ornamental fish trade has increased the worldwide distribution of brine shrimps (Varó et al., 2015). These human-induced activities have accelerated the spread of *Artemia* species across most of the world's

biogeographic regions (Suarez & Tsutsui, 2008). The expanding geographical distribution may impact species diversity and composition of native species (Van Stappen et al., 2007).

1.6 Zooplankton invasions through aquaculture

Zooplankton can be transported over long distances either via natural passive methods such as wind, water currents, and migratory birds or via active methods such as local and international trading of live aquatic organisms (Kolar & Lodge, 2000; Abatzopoulos et al., 2002). The most significant introductory method for non-indigenous aquatic species is associated with importation from other countries. These importations include the less-appreciated vectors such as the aquarium trade, the aquaculture industry and cultivated aquatic plants (Rixon et al., 2005). With the development of the aquaculture industry as well as the ornamental fish trade, people have accelerated the rate and spatial scale of transportation of live organisms or viable eggs around the world to new places where these organisms are not native (Mackie, 2000; Duggan, 2011). Olden et al. (2021) state that the ornamental aquarium trade is the most significant contributor to the spread of aquatic invasive species (e.g., 40 established species out of 100 introduced ornamental fishes in North America have been introduced via the aquarium trade) (Courtenay & Stauffer, 1990). In the scenario of spreading invasive species, the popularity of species plays a vital role; as popularity increases, the importation and the sale of those species increase, creating a high probability of releasing larger numbers of those species into the natural waterways (Duggan et al., 2006)

In the aquarium pet trade, many aquatic organisms are transported intentionally among countries, including fish, large snails and ornamental aquatic plants (Rixon et al., 2005; Chang et al., 2009). On the other hand, some aquatic invertebrates are transported

incidentally via aquarium plants or released into natural habitats with the dumping of water, sediments and detritus (e.g., rotifers, cladocerans, copepods and snails) (Duggan, 2010; Duggan et al., 2018). Patoka et al. (2017) have recorded importations of invertebrates, including nematodes, oligochaetes, snails, insects and zooplankton such as cladocerans, copepods and rotifers, to the Czech Republic with Indonesian water hyacinth importations. In New Zealand, some already established invertebrate species that have been introduced via the aquarium trade include the snail species *Radix auricularia*, *Planorbarius corneus*, *Melanoides tuberculata*, *Physella acuta* and *Lymnaea stagnalis*, while the non-native cnidarian species *Craspedacusta sowerbii* has also been observed in home aquariums (Duggan, 2010). Duggan (2010) confirmed the ongoing risk of incidental zooplankton introductions via the freshwater ornamental fish trade by recording two non-native harpacticoid copepods, *Nitokra pietschmanni* and *Elaphoidella sewelli*, from New Zealand household aquaria, which have not yet been recorded in natural habitats (Duggan, 2010). Aquaculture facilities utilized for farming goldfish (*Carassius auratus*) and grass carp (*Ctenopharyngodon idella*) were responsible for distributing some non-native zooplankton species within New Zealand. For example, recently established *Skistodiaptomus pallidus* and *Daphnia pulex* have been spread through releases of grass carp for macrophyte control in natural waters (Duggan et al., 2014; Branford & Duggan, 2017). Also, the invasive copepod species *S. pallidus* has been recorded from live *Daphnia* samples supplied from a goldfish farm at Te Aroha in the Waikato region as live food to be sold from pet stores throughout New Zealand (Duggan & Pullan, 2017). Traditionally, the pet fish trade functioned primarily through pet or aquarium stores. However, recently, the selling of aquarium fish and their food has become popular via online auction pages (e.g., through AquaBid, eBay or TradeMe) (Olden et al., 2021). Online market dealers have created a worldwide hub with many options for selecting thousands of non-native fish. Due to high demand and fast sales, some

multinational pet fish trading companies such as Petco and Petsmart have started to undertake part of their sales through this online hub (Olden et al., 2021). This has become more common following the COVID-19 pandemic (Olden et al., 2021). Informal marketers enable purchasers to conveniently purchase organisms from sellers globally (Giltrap et al., 2009). This might facilitate long-distance dispersion and allow non-native species to invade a new ecosystem (Lenda et al., 2014).

1.7 Importance of invasive zooplankton

Some invasive species have caused little to no noticeable impact to their new environment (e.g., the invasive cladoceran species *Daphnia lumholtzi*) (Havel et al., 2005; Havel et al., 2015). However, many invasive species can pose a threat to endemic species through competitive and predatory impacts (Duggan et al., 2012; Smits et al., 2013; Kratina et al., 2014; Dexter et al., 2020).

An invasion involves multiple stages, including transportation, introduction, establishment, and the spread of a non-native species (Duggan et al., 2006). Although not all non-native organisms can establish and spread in a new environment, some possess key traits that enable them to adapt successfully and become invasive within an ecosystem (Olden et al., 2021). The characteristic features favour invasiveness include rapid reproduction, highly efficient feeding ability, high growth rates, and high tolerance to unfavourable environmental conditions compared to native species (Ricciardi & Rasmussen, 1998). For example, *A. franciscana* has outcompeted the native *A. salina* and *A. parthenogenetica* in the Mediterranean region by having fast reproduction and efficient filter-feeding ability (Amat et al., 2005). As such, invasive species tend to cause environmental, ecological and economic problems in their new habitat (Pimentel et al., 2005). Once invasive species are established, it

is difficult to irradicate them fully and to return habitats back to the pre-invasion state (Pimentel et al., 2005).

1.8 Threats associated with fish food

The spreading of invasive species via fish food is a potential threat associated with the aquarium trade. For example, Muńoz et al. (2014) and Horváth et al. (2018) emphasise that most of the invasive *Artemia* species found in the Mediterranean region are genetically related to *A. franciscana*, which was exported from the world's main *Artemia* cyst producers, from areas around San Francisco Bay and Great Salt Lake in the USA (Muñoz et al., 2014; Horváth et al., 2018). In New Zealand, pet food stores and online suppliers have been found to sell live fish food, including cultured native *Daphnia thomsoni*, alongside the invasive North American copepod species *S. pallidus* (Duggan & Pullan, 2017). Thus, live fish food is known to be responsible for the movement of invasive zooplankton in New Zealand, though this has not been systematically studied (Duggan, 2011).

1.9 Aim of the study

There have been several recent records of non-native zooplankton invasions in New Zealand, but the transport vectors leading to their introductions are not well understood. Commercially available fish foods containing zooplankton are widely used in home aquariums and the aquaculture industry. These foods are either imported to, or cultured within, New Zealand, and sold as freeze-dried, sun-dried or frozen adults; as capsulated of decapsulated cysts, or as live individuals. I propose that these foods may carry non-native species and thus represent

an invasion risk. As such, this study aims to investigate whether there is a risk of the introduction or spreading of non-native zooplankton via fish food in New Zealand.

Methods

2.1 Types of fish food

Commercially available fish food was obtained as freeze-dried, sun-dried, frozen, capsulated and decapsulated eggs, and live zooplankton (Table 1). Samples were bought from: visiting physical stores; through searches on the New Zealand internet auction site Trademe, using the search terms "*Artemia*", "Brine" "Shrimp", "Zooplankton", "*Daphnia*", "Rotifers", and "*Brachionus*"; and through the internet pages of New Zealand based commercial suppliers.

Freeze-dried *Daphnia* (whole individuals) were obtained that originated from manufacturers in Germany and China, and *Daphnia* labelled as 'sun-dried' was obtained originating from an independent German manufacturer. Frozen *Daphnia* individuals were produced by one company originating from Canada, and live individuals were obtained from five different North Island suppliers.

Artemia was obtained as frozen and freeze-dried adults and as capsulated (the eggs contain dormant, encysted embryos that undergo severe dehydration during processing yet remain capable of hatching quickly upon rehydration) and decapsulated cysts (eggs without shells and obtained through a specialized removal process). Freeze-dried *Artemia* individuals were obtained that originated from two manufacturers, one from China and one from Canada, while freeze-dried brine shrimps were obtained from an individual supplier from China. Capsulated *Artemia* eggs were obtained from manufacturers in the USA, China, and Germany, while the decapsulated eggs were obtained that originated from China. Frozen *Brachionus* were bought originating from a manufacturer in Canada.

Table 1: Types of fish foods, stated containing organisms, and the country of origin

Organism	**Type of fish food**	**Supplier/Brand code**	**Country**
Daphnia	Freeze-dried (FDD)	FDD1	Germany
		FDD2	China
	Sun-dried (SDD)	SDD1	Germany
	Frozen *Daphnia* (FRD)	FRD1	Canada
	Live *Daphnia* (LID)	LID1	Te Aroha/NZ
		LID2	Auckland/NZ
		LID3	Wellington/NZ
		LID4	Hamilton/NZ
		LID5	Wellington/NZ
Artemia	Frozen *Artemia* (FRA)	FRA1	China
		FRA2	China
		FRA3	Canada
		FRA4	Canada
	Freeze-dried (FDB)	FDB1	China
	Capsulated cysts (CCA)	CCA1	USA
		CCA2	China
		CCA3	Germany
	Decapsulated cysts (DCA)	DCA1	China
Brachionus	Frozen (FRB)	FRB1	Canada

2.2 Hatching diapausing *Daphnia* eggs

Many of the freeze-dried and sundried *Daphnia* were observed to carry diapausing eggs (sexually produced eggs, formed under unfavourable environmental conditions entering a state of dormancy and capable of hatching when conditions improve, are enclosed in chitinous shells known as ephippia in their brood pouches). To test for the potential hatching of these eggs, 0.2 g of the two freeze-dried and the one sun-dried *Daphnia* samples were soaked for two hours in Reverse Osmosis (RO) water. Then, the individual *Daphnia* with ephippia were sorted and counted, while observing under a stereo dissecting microscope (Nikon SMZ 645) at 20 × magnification. The sorted individuals were transferred to Petri dishes with 10 mL of distilled water. Next, eggs were removed from the ephippia under the dissecting microscope. The ephippia's outer covering and the soft inner membrane were removed using microdissection forceps and needles. Then, the embryos were transferred using a Pasteur pipette into Petri dishes containing 10 mL of growth medium. The dormant eggs were incubated in three different media, ADaM (Klüttgen et al., 1994) (Appendix A), artificial/synthetic pond water (Hebert & Crease, 1980) (Appendix B), and filtered pond water (filtered through mounted pre-sterilized Whatman GF/C glass microfiber filters, with a nominal 0.45 µm pore size, using a vacuum pump and a Nalgene Analytical Test Filter Funnel). Pond water was obtained from Chapel Lake on the Hamilton campus of the University of Waikato (37°47'17.7"S, 175°18'53.5"E). Sixty eggs from each sample were incubated in the three media types, with 20 eggs in each. Finally, all nine Petri dishes were incubated in a Thermoline Scientific Refrigerated Incubator for four weeks, exposed to a full spectrum long day photoperiod light (16:8) at a temperature of 15 °C (Radzikowski et al., 2017).

2.3 Incubation of *Brachionus* eggs

On return from the store, the frozen *Brachionus* sample was stored in a freezer at -20 ℃ until use. Immediately prior to incubation, the sample was kept at room temperature until the sample was defrosted. Meanwhile, the incubation medium was prepared using RO water, pre-aerated for 24 hours. The eggs were incubated in five 1000 mL beakers, with media having five different salinities; 10 ppt, 15 ppt, 17 ppt, 20 ppt, and 25 ppt, respectively (Gilbert, 2017). The different salinities were obtained by dissolving sea salt (Crystal Sea ® Marinemix, Marine Enterprises International) in the pre-aerated RO water. A YSI Meter Pro 2030 meter was used to measure the salinity of the medium. After preparing the incubating media, 30 mL of the unfrozen *Brachionus* sample was transferred using a micropipette into each beaker separately. An approximate 20 ℃ incubating temperature was obtained using a lamp fitted with an incandescent light bulb adjacent to the setup. The beakers were sealed using parafilm to prevent evaporation, which could cause changes in the salinity.

2.4 Hatching of capsulated and decapsulated *Artemia* cysts

For the hatching of capsulated *Artemia* cysts, glass aquariums (5000-7000 mL) were filled with RO water which was pre-aerated for 24 hours. The salinity of the media was adjusted to 25 ppt by dissolving sea salt (Crystal Sea ® Marinemix, Marine Enterprises International) (Dey et al., 2023). Then, 0.2 g of capsulated eggs were added to the medium. The aquaria were warmed with an incandescent lamp adjacent to the setup (Dey *et al*. 2023). The tank was closed using a glass plate to prevent evaporation which could cause changes in the salinity. For the process of hatching the decapsulated cyst sample, the number of eggs used

was reduced to 0.1 g, as the number of eggs per gram was higher than for the capsulated eggs.

Once hatched, *Artemia* were fed daily with *Spirulina* powder (Essnté Professional Kitchen brand) or instant yeast (Edmonds). The feeding solution was prepared by dissolving *Spirulina* powder or yeast in RO water. In an alternating pattern, six to eight millilitres of food solution was added to one aquarium (for 7000 mL) once per day. Water in the tanks was changed once per month.

2.5 Incubation of eggs from frozen adult *Artemia*

The frozen *Artemia* samples were stored at -20 °C until use. Two grams of the samples were kept at room temperature for one hour to defrost. Then, the samples were transferred into Petri dishes. Fifty eggs from each sample were separated from a pool of eggs made up of all the eggs found in the females' brood sac within the 2 g sample. Egg separation from brood sacs and selection of healthy eggs were undertaken while being observed under a dissecting microscope at 20 × magnification. The incubation medium was prepared for the hatching of commercial cysts, above. The aquaria were warmed by keeping a lamp fitted with an incandescent built adjacent to the setup (Dey et al., 2023).

2.6 Live *Daphnia* samples

Upon arrival at the laboratory, live *Daphnia* samples were filtered through a 40 µm sieve, and material retained on the sieve was preserved in 95% ethanol (final concentration of 70%). Samples were enumerated in 5 mL aliquots using a gridded Perspex counting tray, under a

stereo microscope at 30 x magnification. The animals in the samples were identified using standard identification guides (Shiel, 1995; Chapman et al., 2011). The samples were then labelled and stored in the laboratory refrigerator (4 °C) until prepared for genetic analyses.

2.7 Molecular analysis

2.7.1 Sample collection

Tissue samples of *Daphnia* (freeze-dried, sun-dried, frozen, and those preserved in 70% alcohol), *Artemia* (frozen, freeze-dried, and live *Artemia*), and *Brachionus* eggs and damaged adult body parts were loaded into a microplate to be processed at the Canadian Centre for DNA Barcoding (CCDB). The filling of the microplate was carried out according to the guidelines provided by the CCDB (Appendix C). One individual *Daphnia* was loaded into one microplate well with 95% ethanol, while a nearly 3 mm long tissue sample from *Artemia* was used. Two grams of freeze-dried *Artemia* samples were soaked in separate petri dishes containing RO water for 1 hour. Then, 3 mm-long tissue samples were collected from each petri dish and placed into microplate wells with 95% ethanol, with five replicates per sample.

Two microliters from the *Brachionus* sample containing dead adults and eggs were loaded into one microplate well. The animal tissue samples (95) were given a sample identification code (ID) and the CCDB plate record was submitted to the CCDB. Meanwhile, specimen data and image submission to the Barcode of Life Data (BOLD) Systems was done according to the protocols provided by the BOLD Systems.

2.7.2 DNA extraction, PCR amplification, and DNA sequencing

DNA extraction was performed at CCDB using the glass fiber method (Ivanova et al., 2006). PCR amplification, targeting a 658 bp fragment of the cytochrome c oxidase I (COI) gene with the Platinum® *Taq* DNA polymerase enzyme was conducted using a combination of two forward primers (*LepF1*: 5′-ATTCAACCAATCATAAAGATATTGG-3′ and *MLepF1*: 5′-GCTTTCCCACGAATAAATAATA-3′) and two reverse primers (*LepR1*: 5′-TAAACTTCTGGATGTCCAAAAATCA-3′ and *MLepR1*: 5′-CCTGTTCCAGCTCCATTTTC-3′) (Hajibabaei et al., 2006; Ivanova & Grainger, 2007a; Spiess et al., 2004). PCR recipe for a final reaction volume of 12.5 µL containing 10% trehalose 6.25 µL, ddH_2O 2 µL, 10x buffer 1.25 µL, 50 mM MgCl2 0.625 µL, 10 µM forward primer 0.125 µL, 10 µM reverse primer 0.125 µL, 10 mM dNTPs 0.0625 µL, Platinum® *Taq* DNA polymerase 0.06 µL. Standard conditions for COI gene amplification begin with an initial denaturation at 94°C for 1 minute. This is followed by five cycles of denaturation at 94°C for 30 seconds, annealing at 45-50°C for 40 seconds, and extension at 72°C for 1 minute. Subsequently, 30 to 35 additional cycles are performed, consisting of denaturation at 94°C for 30 seconds, annealing at 51-54°C for 40 seconds, and extension at 72°C for 1 minute. The process concludes with a final extension at 72°C for 10 minutes, followed by a storing temperature at 4°C (Ivanova & Grainger, 2007b).

Dye terminator sequencing was performed in both directions to sequence the COI gene. The sequencing reactions were carried out in a thermocycler under the following conditions: an initial denaturation at 96°C for 2 minutes, followed by 30 cycles of denaturation at 96°C for 30 seconds, annealing at 55°C for 15 seconds, and extension at 60°C for 4 minutes, concluding with an indefinite hold at 4°C (Ivanova & Grainger, 2007c). Excess dye terminators and other impurities from the completed cycle sequencing reactions were removed using Agencourt® CleanSEQ®, a magnetic bead-based purification method

(Grainger & Ivanova, 2007). The purified sequencing products were then eluted from the beads and analysed using the Applied Biosystems 3730xl DNA Analyzer, generating the final sequence reads (Grainger & Ivanova, 2007).

2.7.3 BLAST analysis of *Daphnia* DNA sequences

The raw *Daphnia* sequences (27 paired ab1 files) uploaded to the BOLD Systems by CCDB were downloaded and imported into Geneious v10.2.6. The forward and reverse sequences were aligned using MUSCLE (Edgar, 2004) and trimmed. Consensus sequences were obtained from the alignments and used for subsequent analyses. The NCBI Genomic BLAST online tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to verify the taxonomic classification of the species sequences, and it is summarised in Table 3a, 3b, & 3c.

2.7.4 Phylogenetic Analysis of *Artemia* DNA sequences

Artemia sequences were processed in Geneious and classified using BLAST using the same methods outlined for *Daphnia* (2.7.1). A total of 41 consensus nucleotide sequences from *Artemia* in my study were combined with 18 sequences from NCBI representing the diversity of *Artemia* species. These were aligned using MAFFT in Geneious v10.2.6 (Katoh et al., 2002) and trimmed using ClipKIT (Steenwyk et al., 2020) in smart-gap mode, resulting in an alignment of 531 nucleotides. A maximum likelihood tree was constructed using IQ-TREE Version: 2.2.2.2 (Nguyen et al., 2015) with ModelFinder (Kalyaanamoorthy et al., 2017) using the jmodel test and 1000 bootstrap replicates and outgroups set as *Parartemia contracta* and *P. longicaudata* (Appendix D). The resulting tree was produced using the Tamura-Nei model with empirical base frequencies and a gamma distribution with four rate

categories (TN+F+G4, $\alpha = 0.477$). Tree visualization, branch concatenation and annotations were performed with Evolview v3 (Steenwyk et al., 2020).

Results

3.1 Experimental results and observational data for *Daphnia* containing fish food samples

3.1.1 Hatching of diapausing *Daphnia* eggs

No eggs found in the brood pouches of the freeze-dried and sun-dried *Daphnia* could be hatched using any of the three different incubating media (ADaM, artificial pond water, and filtered pond water).

3.1.2 Species observed in live *Daphnia* samples

The morphological-based identification data and the BLAST analysis data for *Daphnia* were the same. *Daphnia pulex* s.l. was recorded from Te Aroha (LID1), Hamilton (LID4), and Wellington (LID5) samples (Table 3a & 3b). The Auckland sample (LID2) and the Wellington (LID3) sample contained *D. carinata* s.l. (Table 3a & 3b). Other zooplankton taxa were recorded from live samples, including cladocerans, ostracods, copepods, rotifers, and copepod nauplii (Table 2).

3.1.3 DNA analysis of fish feed containing *Daphnia*.

Freeze-dried *Daphnia* samples obtained from two different manufacturers from Germany and China contained the same *Daphnia* species, *Daphnia magna* Straus,1820. The sun-dried

sample from Germany and the frozen *Daphnia* sample obtained from a Canadian manufacturer also contained *Daphnia magna* (Table 3b & Table 3c)

Table 2: Species identified, number of individuals per sample, and supplying location of zooplankton from live samples.

Sample	**Species**	**Sample volume**	**No of individuals**	**Area/City of the supplier**
LD1	*Daphnia pulex* s.l.	2 L	**601**	Te Aroha
	Ostracods		43	
	Chydorus sp.		17	
	Mesocyclops australiensis		11	
	Platyias quadricornis		1	
	Nauplii larvae		1	
LD2	*Daphnia carinata* s.l.	50 mL	**64**	Auckland
	Chydorus sp.		86	
	Chironomid larvae		2	
LD3	*Daphnia carinata* s.l.	245 mL	**170**	Wellington
	Chydorus sp.		46	
	Cyclopoid copepods (juveniles)		2	
	Nauplii larvae		1	
LD4	*Daphnia pulex* s.l.	560 mL	**151**	Hamilton
	Eucyclops serrulatus		10	
	Mosquito larvae		1	

LD5	*Daphnia pulex* s.l.	1150 mL	**306**	Wellington
	Ostracods		12	
	Mosquito larvae		10	
	Chironomid larvae		4	

Table 3 a: BLAST results (Max Score, Total Score, Query Cover, E-value, Percent Identity, Accession Length, Accession number, and locality of closest matching sequence) from molecular analysis of fish food containing *Daphnia*.

Sample	Scientific name	Accession Number of the closest match	Max Score	Total Score	Query Cover	E-value	Per cent Identity	Accession Length	Locality of the closest matching sequence
LID1	*Daphnia pulex*	LC632384.1	1190	1190	93%	0	98.5	15321	Japan
LID1	*Daphnia pulex*	LC632384.1	1092	1092	98%	0	92.94	15321	Japan
LID1	*Daphnia pulex*	LC632384.1	1164	1164	95%	0	96.46	15321	Japan
LID1	*Daphnia pulex*	LC632384.1	1227	1227	96%	0	99.26	15321	Japan
LID2	*Daphnia carinata* s.l.	KU876922.1	1146	1146	95%	0	97.86	654	New Zealand
LID2	*Daphnia carinata* s.l.	KU876922.1	1147	1147	95%	0	98.16	654	New Zealand
LID2	*Daphnia carinata* s.l.	KU876922.1	1142	1142	95%	0	98.01	654	New Zealand
LID2	*Daphnia carinata* s.l.	KU876922.1	1157	1157	97%	0	98.18	658	New Zealand
LID2	*Daphnia carinata* s.l.	KU876922.1	1122	1122	99%	0	96.81	658	New Zealand
LID3	*Daphnia carinata* s.l.	KU876922.1	1170	1170	95%	0	98.63	658	New Zealand
LID4	*Daphnia pulex*	LC632384.1	1186	1186	96%	0	98.49	15321	Japan

Table 3 b: BLAST results (Max Score, Total Score, Query Cover, E-value, Percent Identity, Accession Length, Accession number, and locality of the closest matching sequence) from molecular analysis of fish food containing *Daphnia*.

Sample	Scientific name	Accession Number of the closest match	Max Score	Total Score	Query Cover	E-value	Per cent Identity	Accession Length	Locality of the closest matching sequence
LID4	*Daphnia pulex*	LC632384.1	1184	1184	95%	0	98.49	15321	Japan
LID4	*Daphnia pulex*	LC632384.1	1195	1195	94%	0	98.21	15321	Japan
LID4	*Daphnia pulex*	LC632384.1	1192	1192	95%	0	98.79	15321	Japan
LID4	*Daphnia pulex*	LC632384.1	1205	1205	96%	0	99.24	15321	Japan
LID5	*Daphnia pulex*	LC632384.1	1199	1199	96%	0	97.66	15321	Japan
LID5	*Daphnia pulex*	LC632384.1	1205	1205	97%	0	98.8	15321	Japan
LID5	*Daphnia pulex*	LC632384.1	1214	1214	96%	0	99.55	15321	Japan
LID5	*Daphnia pulex*	LC632384.1	1190	1190	95%	0	98.49	15321	Japan
LID5	*Daphnia pulex*	LC632384.1	1177	1177	99%	0	98.04	15321	Japan
FDD1	*Daphnia magna*	MH683667.1	1227	1227	99%	0	99.41	15765	Switzerland
FDD1	*Daphnia magna*	MH683667.1	1212	1212	100%	0	98.39	15765	Switzerland

Table 3 c: The BLAST results (Max Score, Total Score, Query Cover, E-value, Percent Identity, Accession Length, Accession Number and locality of the closest matching sequence) from molecular analysis of fish food containing *Daphnia* and *Brachionus.*

Sample	Scientific name	Accession Number of the closest match	Max Score	Total Score	Query Cover	E-value	Per cent Identity	Accession Length	Locality of the closest matching sequence
FDD1	*Daphnia magna*	MK236272.1	1205	1205	100%	0	99.54	658	China
FDD1	*Daphnia magna*	MH683667.1	1216	1216	99%	0	99.41	15765	Switzerland
FDD1	*Daphnia magna*	MH683667.1	1221	1221	99%	0	99.85	15765	Switzerland
FDD2	*Daphnia magna*	MH683667.1	1114	1114	100%	0	98.57	15765	Switzerland
FDD2	*Daphnia magna*	MH683667.1	1114	1114	100%	0	98.57	15765	Switzerland
SDD1	*Daphnia magna*	MH683667.1	1225	1225	97%	0	100	15765	Switzerland
SDD1	*Daphnia magna*	MH683667.1	1214	1214	98%	0	99.4	15765	Switzerland
SDD1	*Daphnia magna*	MH683667.1	1230	1230	96%	0	98.97	15765	Switzerland
SDD1	*Daphnia magna*	MH683667.1	1206	1206	97%	0	99.55	15765	Switzerland
SDD1	*Daphnia magna*	MH683667.1	1206	1206	96%	0	99.25	15765	Switzerland
FRD1	*Daphnia magna*	NC_026914.1	1085	1085	96%	0	95.14	14948	China
FRD1	*Daphnia magna*	KM244710.1	1149	1149	100%	0	96.74	14377	China
FRB1	*Brachionus manjavacas*	MW559989.1	22666	22666	100%	0	100.00	12274	Korea

3.2 Experimental Analysis of Food Samples Containing *Artemia*

3.2.1 Hatching of capsulated and decapsulated *Artemia* cysts

The hatching experiments for capsulated and decapsulated *Artemia* cysts from all suppliers were successful. The BLAST analysis indicated that the capsulated (CCA) samples from USA and German suppliers contained *Artemia franciscana*, while the Chinese supply contained *A. parthenogenetica* (Table 4). The decapsulated (DCA) cysts from a Chinese manufacturer hatched into *A. parthenogenetica* (Table 4; Figure 1).

3.2.2 Incubation of eggs from frozen adult *Artemia*

The incubated eggs obtained from frozen *Artemia* from the two Chinese manufacturers (FRA1 and FRA2) and two Canadian manufacturers (FRA3 and FRA4) could not be hatched. The BLAST analysis of frozen samples revealed the presence of *Artemia franciscana* in all of the frozen samples. The freeze-dried sample (FDB) originating from China also contained *Artemia franciscana* (Table 4 and Figure 1). There were five *A. franciscana* haplotypes out of the 31 *A. franciscana* sequences in the tree and only one haplotypes from the 9 sequences in the *A. parthenogenetica* group. Of the 41 *Artemia* sequences, 31 were classified as *A. franciscana*, 9 as *A. parthenogenetica* / *A. tibetiana* / *A. urmiana* and one as *A. sinica* (Figure 1).

Table 3: BLAST analysis data of the recorded *Artemia* spp. from fish feed, Freeze-dried (FDD), Frozen (FRA), and Live *Artemia* from Capsulated (CCA) and Decapsulated cysts (DCA) and country or origin of samples.

Type of fish feed	**Sample**	**Species**	**Country of Origin**
Freeze-dried	FDD	*A. franciscana*	China
Frozen	FRA1	*A. franciscana*	China
	FRA2	*A. franciscana*	China
	FRA3	*A. franciscana, A. sinica*	Canada
	FRA4	*A. franciscana*	Canada
Capsulated cysts	CCA1	*A. franciscana*	USA
	CCA2	*A. parthenogenetica*	China
	CCA3	*A. franciscana*	Germany
Decapsulated cysts	DCA1	*A. parthenogenetica*	China

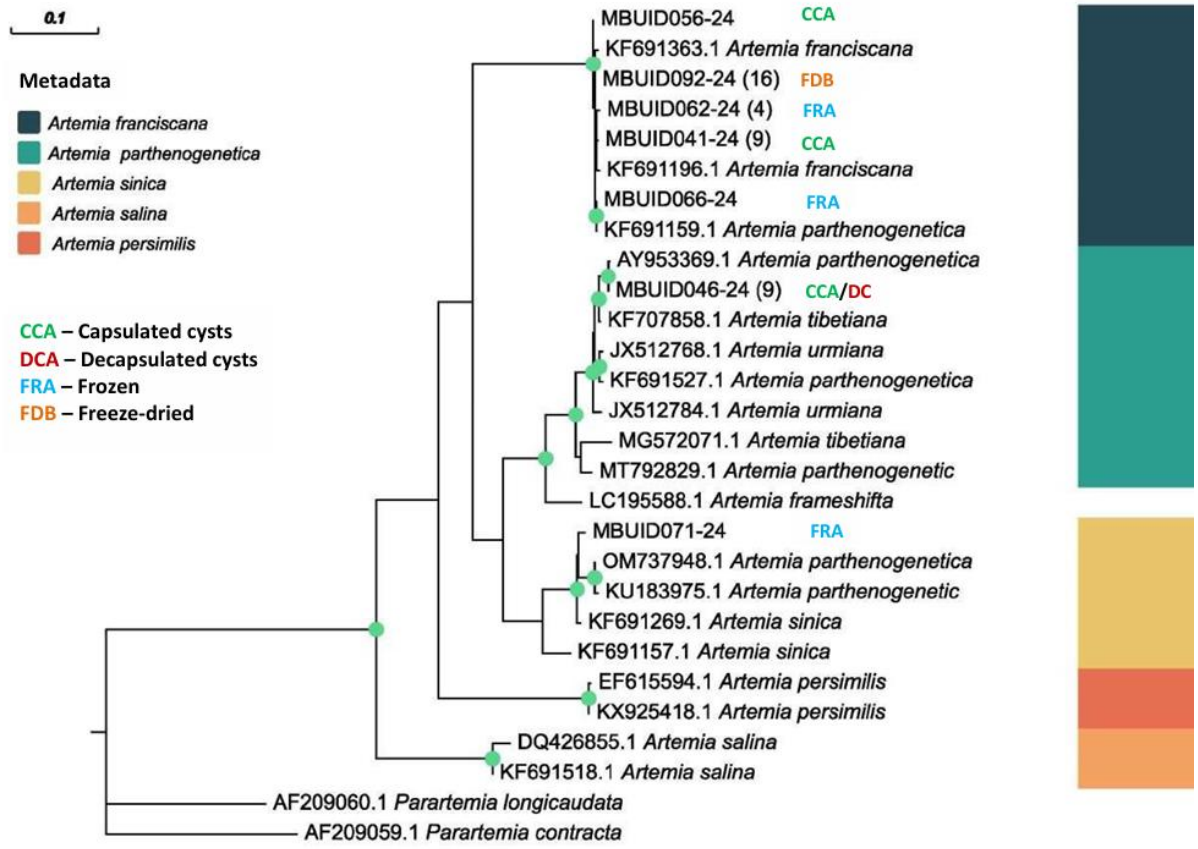

Figure 1: Phylogenetic tree of *Artemia* species constructed using the Maximum likelihood method based on the COI mitochondrial gene and was constructed using IQ-TREE Version:2.2.2.2. with Model Finder using the jmodel test and 1000 bootstrap replicates. *Paratemia contracta* and *P. longicaudata* were used as outgroups to root the tree.

3.3 Hatching of *Brachionus* eggs

The frozen *Brachionus* sample manufactured in Canada comprised eggs and damaged adult individuals. No eggs were hatched following incubation in different salinities (10 PSU, 15 PSU, 17 PSU, 20 PSU, and 25 PSU). The BLAST analysis indicates that the animal tissues included in the sample belonged to *Brachionus manjavacas* (Table 3c).

Discussion

4.1 Invasion risks and their management strategies

When considering live *Artemia*, this study indicated that viable capsulated and decapsulated eggs of *Artemia parthenogenetica* have been imported to New Zealand. According to the Import Health Standards for Fish food and fish baits from all countries (Ministry for Primary Industries, 2023), it is prohibited to import any *Artemia* species other than *A. salina* and *A. franciscana*. Due to the importation of cysts of parthenogenetic lineages, there is a risk of colonisation of this taxon. For example, parthenogenetic lineages, which originated from the old-world sexual species *A. sinica* and *A. urmiana*, have spread into the western Mediterranean region (Sainz-Escudero et al., 2021; Rode et al., 2022). Similarly, Asian parthenogenetic lineages have replaced native *A. salina* in the Iberian Peninsula, south-west Europe (Pang et al., 2024). Moreover, some parthenogenetic strains possess specific biotopes that enable them to outcompete invasive *A. franciscana* (Pinto et al., 2014). Thus, it is possible for parthenogenetic lineages to establish in habitats of *A. franciscana*, leading to the eradication of existing *A. franciscana* populations. Alternatively, there is potential for co-existence of the parthenogenetic strains with existing *A. franciscana* populations. In Asia, where there are approximately 530 sites with *Artemia* populations, *A. franciscana* has successfully established populations in many locations. In some cases, *A. franciscana* co-exists with other sexual *Artemia* species or parthenogenetic populations, as confirmed by molecular analyses (Van Stappen et al., 2007; Eimanifar et al., 2014).

In New Zealand, there has been only one identified population of *Artemia* to date, in Lake Grassmere. Salt production in New Zealand commenced in 1954 following the identification

of a suitable location for salt pans, in Lake Grassmere in the Marlborough region. This shallow lagoon experiences high solar radiation and strong winds while remaining isolated from the major river, the Awatere River, making it the only suitable site for salt production in the country. If a non-native *Artemia* species were to become established in New Zealand, Lake Grassmere would be one of the few known potential habitats with appropriate environmental conditions (Wear et al., 1986). Other than Lake Grassmere, there is one inland salt lake, Lake Sutton in Central Otago, with a salinity ranging from 11-15 PSU (Craw & Beckett, 2004), which may have the potential to be an appropriate habitat for *Artemia*. For establishment to occur, viable *Artemia* cysts or live stages would need to be transported to the lake. Given that *Artemia* survival rate is only about 30% in freshwater (Soundarapandian & Saravanakumar, 2009), their survival after entering stormwater discharge is highly improbable. Additionally, *Artemia* cannot persist in marine environments for extended periods (Soundarapandian & Saravanakumar, 2009), meaning that even if stormwater carried live individuals or cysts to the ocean, the likelihood of their successful transfer to Lake Grassmere or other potential sites remains extremely low. However, in this study, successful hatching of *Artemia* cysts occurred below hypersaline conditions, such as at 25 PSU, creating a probability of establishment if cysts were to be dispersed via stormwater into saline coastal ponds (Marshall & Duggan, 2024). Consequently, the unintentional introduction and establishment of a new *Artemia* population in Lake Grassmere is highly unlikely.

According to the phylogenetic analysis, *Artemia* species hatched from the capsulated and decapsulated eggs belonged to *A. franciscana* and *A. parthenogenetica*. The capsulated cysts of *A. franciscana* were mainly obtained from the USA and Germany, indicating that *A. franciscana* is the dominating species in the American and European trade. The capsulated and decapsulated cysts of *A. parthenogenetica* were obtained from manufacturers from China. In the phylogenetic tree, *A. parthenogenetica* is closely related to old-world sexual

species such as *A. tibetiana*, *A. urmiana* and *A. sinica* (Sainz-Escudero et al., 2021). Moreover, *A. frameshifta* is now considered a synonym for *A. urmiana*, which is why it has clustered with the latter clade of *A. parthenogenetica* (Maccari et al., 2013). Studies conducted by Asem et al. (2016), based on three mitochondrial genes, found that parthenogenetic lineages of *Artemia* belong to a polyphyletic group, suggesting that diploid and tetraploid lineages have originated from *A. urmiana* and *A. sinica*, while tri and pentaploid lineages have arisen from diploid and tetraploid lineages respectively. This taxonomic complexity makes *Artemia* identification more complicated and traditional morphological-based taxonomic identification cannot be relied on. According to the phylogenetic tree, parthenogenetic strains can be considered to be closely related.

Some *Artemia* species hatched did not belong to either *A. franciscana* or *A. salina*; the latter were, in fact, not found at all, despite being listed as allowable. According to the New Zealand Inventory of Biodiversity, *Artemia franciscana* is the only *Artemia* species known to have an established population in New Zealand (Gordon, 2010). However, according to the Import Health Standards for Fish food and fish bait from all countries (Ministry for Primary Industries, 2023), A. *franciscana* and *A. salina* are allowed to be imported, likely because both species were considered to occur in New Zealand prior to 29 July 1998, as per the Biosecurity Act requirements (as at 23 December 2023). Since *Artemia* was not found in New Zealand prior to 1974, *Artemia franciscana* was likely introduced from individuals originating from Great Salt Lake, Utah, USA (Knight, 1974). As such, the species present in Lake Grassmere is likely not be *Artemia salina*, but most probably represents *A. franciscana* (Bowen & Sterling, 1978; Haslett & Wear, 1985). Thus, *A. salina* is not considered to be established in the country, and its inclusion in the Import Health Standards for Fish food and fish bait from all countries (Ministry for Primary Industries, 2023) may require reconsideration to ensure alignment with biosecurity policies. Nevertheless, the *Artemia*

population in Lake Grassmere has never been genetically tested, making it unclear as to the taxonomic status of that population.

Attention to non-indigenous species commonly comes after they have established in a new environment. After establishing a non-indigenous population, however, it is difficult or impossible to eradicate them (Rixon et al., 2005). According to Duggan (2010), the aquarium trade has likely contributed to numerous invasions of New Zealand's freshwater ecosystems. While most recorded non-native species have already established themselves, the trade still presents a potential risk for further establishment and spread. Therefore, consideration of best management practices in the aquarium trade would be the best solution to prevent the establishment of non-indigenous *Artemia* species in New Zealand aquatic systems.

Sellers of live *Daphnia* were distributed widely around the North Island. Native *Daphnia carinata* s.l. (which, due to their matching COI sequences with New Zealand populations, will be comprised of *D. thomsoni*, *sensu* Burns et al. 2017) and invasive *Daphnia pulex* were found to be sold and distributed as live fish food in New Zealand. The trade of non-native *Daphnia pulex* may have facilitated their movement, posing a risk of further spread, particularly when buyers use them for outdoor pond cultures instead of as feed for fish. An increase in the number of individual breeders cultivating this invasive species is likely to accelerate its dispersal rate and heighten the risk of colonization in new environments. Additionally, improper disposal of waste from culture tanks into natural waters may lead to the unintended release of non-native *D. pulex*, increasing the risk of their establishment in ecosystems where they are not yet present. According to Duggan (2010), to minimize the risk of invasion by incidental invertebrate fauna through the aquarium trade, it is important to focus on improving border security measures and homeowner management practices. Furthermore, aquarium owners should be informed about the potential risks of incidental fauna in their tanks and be encouraged to dispose of tank washings on their lawns or gardens

rather than down drains as a best practice (Duggan, 2010). Moreover, if wastewater from the aquarium trade is discharged directly into natural waterways via stormwater drains, it may contain diapausing eggs produced by *Daphnia* in a dormant embryonic state (Vandekerkhove et al., 2005). These eggs have the potential to hatch when favourable environmental conditions arise, posing a risk of unintended species introductions (Cambronero & Orsini, 2018). Therefore, to prevent the further spread of invasive *Daphnia* species through the live fish food trade, regular sampling for species taxonomic identification, limiting the use of non-native species, and strict maintenance protocols at *Daphnia* culture facilities are essential.

Courtenay and Stauffer (1990) stated that any aquarium fish could potentially be released into the wild at some point. This primarily occurs when aquarium owners grow tired of their fish, the fish outgrow indoor tanks, or individuals believe that releasing unwanted pet fish into outdoor ponds or natural waterways is a more humane alternative to euthanasia (Courtenay, 2000). However, when releasing fish to another location, fish are often carried in containers containing water from their original aquarium. If the original aquarium is comprised of any invasive zooplankton (i.e., live fish feed *Daphnia pulex*), there is a tendency to transport them with the released fish species. This practice poses a risk of introducing non-indigenous zooplankton into the receiving water body, potentially leading to ecological contamination. Therefore, to prevent future invasions caused by such methods, strategies should be implemented to reduce propagule supply. These may include educating individuals on the potential ecological and legal consequences of releasing organisms and providing options for returning unwanted organisms to aquarium stores (Courtenay & Taylor, 1986). If non-indigenous species are introduced into an open outdoor pond, aquatic birds can serve as vectors, facilitating their dispersal to natural water bodies and potentially acting as stepping stones in their establishment (Duggan, 2010).

To ensure effective management, regular monitoring should be implemented by management authorities to identify *Daphnia* species being sold by all commercial aquariums and home-scale *Daphnia* breeding facilities. This program should focus on the taxonomic identification of traded species as well as non-traded species associated with breeding and rearing systems. This action should facilitate stopping the trading of non-native *Daphnia* but keep the trading of native *Daphnia* species.

4.2 Hatching experiments of *Artemia* cysts, *Daphnia* diapausing eggs and *Brachionus* eggs

The *Daphnia* diapausing eggs from both freeze-dried and sun-dried fish food failed to hatch in any of the three incubation media: ADaM, artificial (synthetic) pond water, and filtered pond water. Further, no *Brachionus* eggs were successfully hatched.

During the processes of freeze-drying and sun-drying, there is a high likelihood of damage to the embryos, which can lead to the loss of viability in diapausing *Daphnia* eggs, *Artemia* cysts, and *Brachionus* eggs (Balompapueng et al., 1997). Thus, freeze-dried eggs have been found not to hatch elsewhere (Kornicker & Sohn, 1979; Angell & Hancock, 1989; Balompapueng et al., 1997). Consequently, it can be concluded that neither freeze-dried nor sun-dried *Daphnia* eggs, nor frozen *Artemia* eggs, pose a risk of hatching due to the detrimental effects of these preservation methods on embryo viability (Angell & Hancock, 1989), and thus seemingly pose no invasion risk.

4.3 Application of DNA barcoding

Out of the 95 samples analysed, 13 did not yield any DNA while 7 out of the remaining 82 did not yield high-quality DNA sequences following DNA barcoding (Appendix E). This may be attributed to DNA degradation in the samples. For the *Brachionus* sample, only fragmented body parts of adult *Brachionus* and eggs were available. Out of the five sub-samples of the *Brachionus* sample analysed, four did not yield high-quality sequences. The BLAST analysis of the remaining high-quality sequence indicates that the animal tissues included in the sample belonged to *Brachionus manjavacas*, from the *B. plicatilis* species complex. DNA extraction from *Brachionus* eggs can be particularly challenging, which may have affected the sequencing results.

Overall, out of all *Daphnia* freeze-dried samples, three samples (MBUID031, 033, 034, and 095) did not produce high-quality DNA sequences in the BLAST analysis. Similarly, six out of *Artemia*, *Daphnia* and *Brachionus* frozen samples (MBUID075, 081, 082, 083, 085, and 089) also had poor DNA sequencing results. However, four out of these six frozen samples contained *Brachionus* specimens, mostly with eggs, which may have made DNA extraction more difficult as discussed above. Since many freeze-dried samples failed to provide quality DNA sequences, this suggests that freezing is a more reliable method for preserving DNA over the long term (Safarikova et al., 2021). The likelihood of errors occurring during DNA extraction, PCR amplification, or DNA sequencing is minimal, as all other samples processed using the same protocol yielded high-quality DNA sequences.

A maximum likelihood tree is an effective tool for organizing and classifying organisms and species based on their DNA sequences. In this study, a 658-bp fragment from the 5′ region of the COI gene of *Artemia*, *Daphnia* and *Brachionus* was amplified. The phylogenetic tree constructed from the COI gene sequences identified distinct clades corresponding to four

species: *A. franciscana*, *A. parthenogenetica*, *A. sinica*, and *A. salina*. Nevertheless, one *A. parthenogenetica* appears in the clade, which is otherwise *A. franciscana*; this is likely to represent a misidentification by the submitters of this sequence. The tree topology not only supports the classification of these species but also offers new insights into their evolutionary relationships. In the phylogenetic tree, *A. parthenogenetica*, *A. tibetiana*, *A. urmiana*, and *A. frameshifta* formed a single clade. That is because *A. parthenogenetica* populations share a close evolutionary relationship with sexual species from the Old World, such as *A. sinica*, *A. urmiana* and *A. tibetiana* (Maccari et al., 2013; Asem et al., 2016). Furthermore, *A. frameshifta* and *A. urmiana* share a close evolutionary relationship (Sainz-Escudero et al., 2021), which explains the placement of *A. frameshifta* within the clade that includes *A. urmiana*. Additionally, *A. parthenogenetica* and *A. sinica* form a separate clade. These two clades are closely related, indicating a high degree of genetic similarity between them.

4.4 Study limitations, implications and future research

This study encountered some practical constraints. One major limitation was the inability to obtain *Daphnia* samples from the South Island due to transport restrictions of aquatic organisms between the North and South Islands (Conservation Act, 1987). Additionally, the extended courier delivery time made it challenging to receive live *Daphnia* through courier services, particularly from geographically distant locations such as Wellington. The prolonged transit time also increased the risk of predation on rotifers and other incidental fauna by predatory cyclopoid copepods within the samples, which can affect the presence-absence data of zooplankton species within the sample.

Further studies should be carried out to confirm the identity of the *Artemia* species present in the Lake Grassmere salt ponds. Also, a survey on internet auction sites should be undertaken to obtain data on the selling and buying locations to assess transportation risks associated with selling live *Daphnia* as fish food. Management authorities should strengthen border regulations to prevent the import of non-native species into New Zealand, including mandating the labelling of species names on packages. Additionally, a registration system should be established under a government authority, such as the Ministry for Primary Industries, to regulate live fish food sellers. Finally, it should be ensured that native *Daphnia* species are being sold by breeders rather than non-native species.

4.5 Summary

In summary, freeze-dried, sun-dried and frozen fish food did not contain viable eggs. Both capsulated and decapsulated *Artemia* cysts were viable. While capsulated eggs produced both sexual and parthenogenetic lineages, decapsulated eggs produced only parthenogenetic lineages. *Artemia parthenogentica* produced by the capsulated and decapsulated cysts supplied by Chinese suppliers is not a legal species for importation into New Zealand.

Live *Daphnia* samples comprised native *D. thomsoni* and invasive *D. pulex*. These findings suggest that some live foods containing *Artemia* and *Daphnia* can be a threat to New Zealand biodiversity and ecosystem health particularly through the rise in the use of online suppliers allowing widespread distribution. *Daphnia pulex* may be a threat to North Island ecosystems if it spreads beyond its current range via live fish food.

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Appendices

Appendix A – Aachener Daphnien Medium (ADaM)

(Klüttgen et al., 1994)

(An artificial freshwater for the culture of zooplankton)

Synthetic sea salt (Wimex hw Meersalz Bioelemente) * - 0.333 g L^{-1}

$CaCl_2$-solution, 0.8 mol L^{-1} (117.6 g L^{-1} $CaCl_2 . 2H_20$) - 2.3 ml L^{-1}

$NaHCO_3$-solution, 0.3 mol L^{-1} (25.2 g L^{-1} $NaHCO_3$) - 2.2 ml L^{-1}

SeO_2-solution, 0.013 mol L^{-1} (1.4 g L^{-1} SeO_2) - 0.1 ml L^{-1}

* Wiegandt GmbH & Co.KG, Sterkenhofweg 13, 47807 Ktefeld, Germany; because the sea salt is hygroscopic, it is stored in an exsiccator to keep it dry.

Appendix B – Artificial/Synthetic Pond Water

(Herbert and Crease, 1980)

$NaHCO_3$	- 48 mg
$CaSO_4.2H_2O$	- 38 mg
$MgSO_4$	- 30 mg
KCl	- 0.5 mg
Distilled water	- 1 L

Appendix C – Microplate filling and data submission instructions

This protocol was provided by the Canadian Centre for DNA Barcoding (CCDB).

https://ccdb.ca/site/wp-content/uploads/2019/07/Instructions_DNA.pdf

A – Container Description - Microplate

Each **microplate** contains 96 sampling wells that are arranged in an 8×12 format. The sampling array starts with well **A01**. Well **H12** should be **left empty** as a negative control, so each plate accommodates 95 tissue samples. The sampling procedure is described in detail in section G.

Each plate is individually numbered with a unique barcode (**CCDB Number**), which should be entered into the corresponding CCDB Plate Record (see section H).

B – Digital Specimen Data Requirements

Prior to molecular analysis at the CCDB, accompanying data must be submitted in a compliant format via two different channels: the CCDB and the **Barcode of Life Data Systems (BOLD)**.

1. The **CCDB Plate Record** named **CCDB-00000_Record.xls**, is emailed to the recipient and used to record the location of samples in the corresponding sample container(s). Each sample must be assigned a **Sample ID**, which is a unique identifier connecting the sample with its source specimen. See section H for more details. Each container will have a corresponding plate record and up to 10 plate records can be included in the CCDB Plate Record file.

2a. A **BOLD Specimen Data Submission** is the first step in the process of creating records on BOLD. There will be one specimen data submission for each batch of containers. For more details on the specimen data submission protocol, please refer to the BOLD handbook accessible through this link: http://www.boldsystems.org/index.php/resources/handbook?chapter=3_submissions.html&section=data_submissions

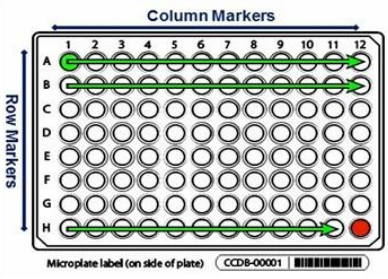
Note: The 'Sample ID' field within this specimen data spreadsheet should be identical (including letter case) to the Sample IDs entered in the CCDB Plate Record and without any duplications.

2b. A **BOLD Specimen Image Submission** is an additional requirement for some analytical services (see http://ccdb.ca/pricing/ for details) and should complement the specimen data submission. For details on the image submission protocol, please refer to BOLD handbook accessible through this link: http://www.boldsystems.org/index.php/resources/handbook?chapter=3_submissions.html&section=image_submissions

C – Microplate Orientation and Addition of Ethanol

Position the microplate on a flat surface with the CCDB plate label facing towards you and with well **A01** positioned at the **TOP LEFT** corner.

IMPORTANT: Add ethanol



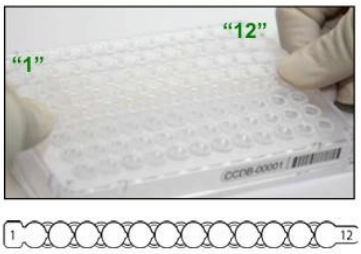
Sampling wells should be pre-filled with **30 µl** of 95-100% **ethanol (not provided with the kit)** using a multi-channel pipettor. If a pipettor is not available, add **one drop** of **ethanol** to each well using an eyedropper just prior to sampling. Always wear gloves when handling the microplate.

Note: Do not add excess ethanol. If the samples are compact and were previously fixed in ethanol (e.g. moist vertebrate muscle tissue), then the addition of ethanol is optional.

Note: Tissue that has not been dried or preserved should not be sampled into a microplate.

IMPORTANT: Never use ethanol if tissue was fixed or stored in dimethyl sulfoxide (DMSO)!

D – Strip Cap Placement



Before proceeding with tissue sampling, place the cap strips (supplied with the submission package) over all rows of wells to avoid contamination. Observe the orientation of cap strips: markers "1" and "12" should match the corresponding columns of the plate.

Note: If sampling immediately, do not fasten caps tightly. If not sampling immediately, seal properly.

When sampling, remove cap strips one at a time and fasten them back when paused, or after finishing each row.

E – Recommended Tissue Size

Below are some examples of recommended tissue sizes for sampling into microplates:

- **Small insect:** whole leg, antenna — >5 mm length
- **Large insect:** tibia or femur only — >2 mm length
- **Vertebrate/invertebrate:** muscle — ca. 1 mm^3 volume or 1 mm diameter
- **2-dimensional tissue:** skin/body wall — ca. 2-4 mm diameter
- **Minute invertebrate:** one whole specimen — ca. <3 mm length

Do not place excessive tissue into the sampling wells - this may inhibit DNA extraction. If the sample exceeds the recommended dimensions, subdivide it into fragments to obtain the right amount for the well.

Avoid sampling from body parts containing scales, hairs or bristles.
Avoid sampling from digestive tracts or from areas which may have been in contact with digestive tract contents or other contaminants.

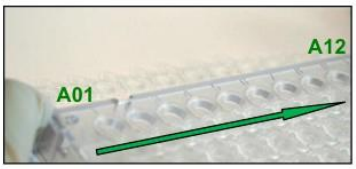
F – Forcep Sterilization Between Samples

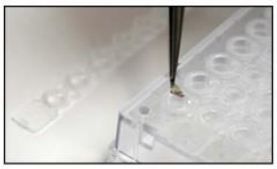
Before proceeding to the next sample, ensure that no residual tissue remains on the forceps. Rinse in 95% ethanol then wipe with a clean kimwipe or tissue.

When the work environment permits, use flame (e.g. for dry insects) or bleach/specialized detergent such as Eliminase (e.g. for vertebrate tissue) to sterilize your sampling tools.

Note: If using bleach or detergent, make sure that all chemicals are completely removed from the tools by thoroughly rinsing in distilled water before the next sampling round, to avoid DNA degradation.

G – The Tissue Sampling Process






Start the tissue sampling process with **A01** (row 1) and proceed to **A12** (left to right). Sample or subsample the recommended amount of tissue from only one specimen with fine forceps (as shown above) and place it into the current sampling well. Confirm that the tissue remains inside the well.

Once complete, enter the corresponding **Sample ID** into the **CCDB Plate Record** (section H). Next, proceed with sampling into **A02**, progressively moving towards **A12**.

When row A is complete, replace the cap strip and seal firmly. Proceed to row B filling from left to right (B01-B12). Repeat this process until all 8 (A-H) rows are filled.

Note: Do not leave empty wells in the middle of the plate. NEVER remove and replace samples.

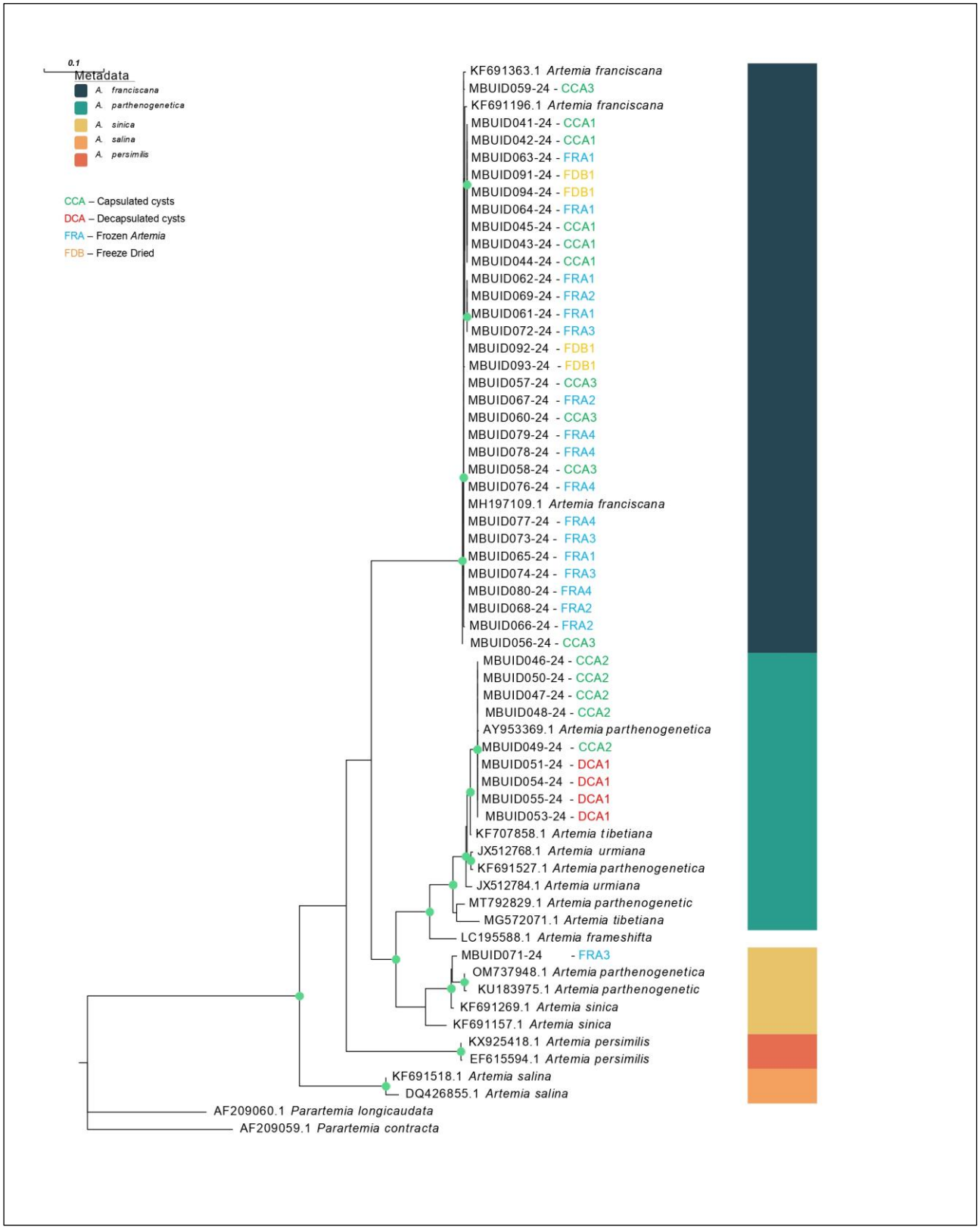
Note: Do not place any foreign objects (e.g. labels) into sampling wells.

IMPORTANT: Do not fill the last well, H12. It should be left empty as a negative control.

Once the plate is filled with samples, ensure that all cap strips are pressed firmly into the wells. Ensure the correct amount of fixative and/or tissue has been sampled from only one specimen into each well by examining the microplate from underneath.

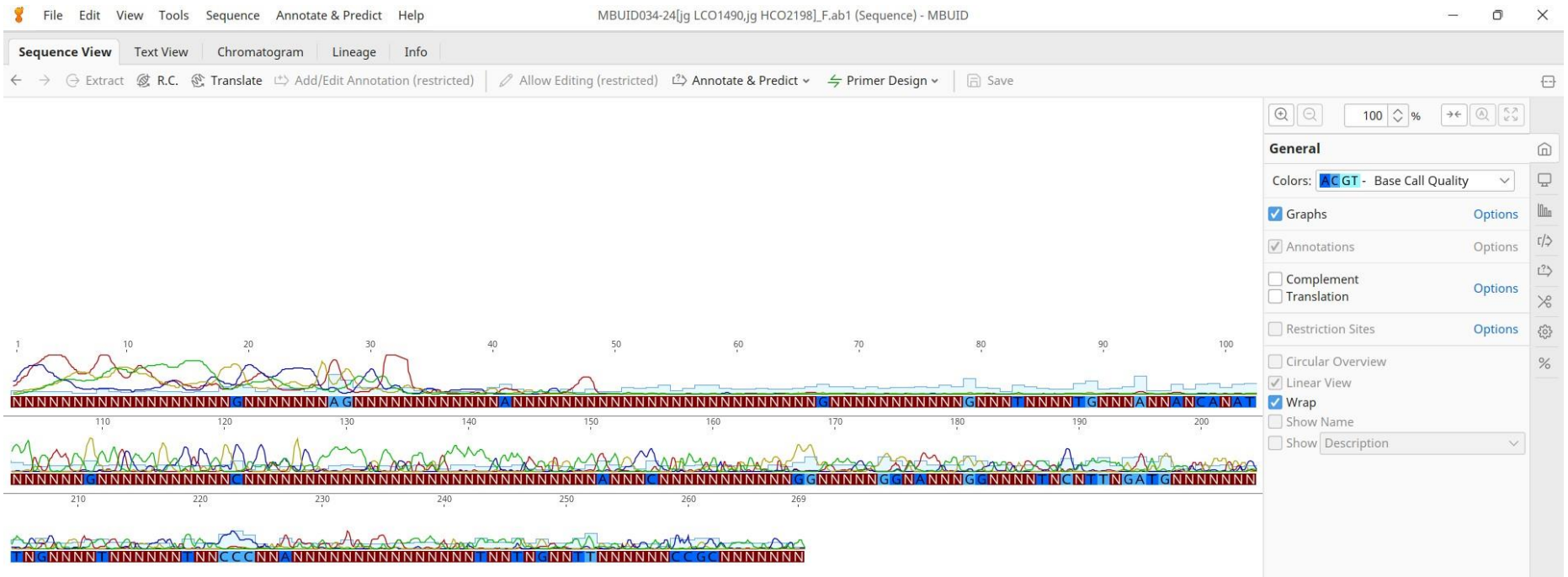
Note: Tissue samples sent in the container(s) may be destroyed unless voucher recovery is requested. Please notify the CCDB if voucher recovery is necessary.

Appendix D – The phylogenetic tree of recorded *Artemia* species



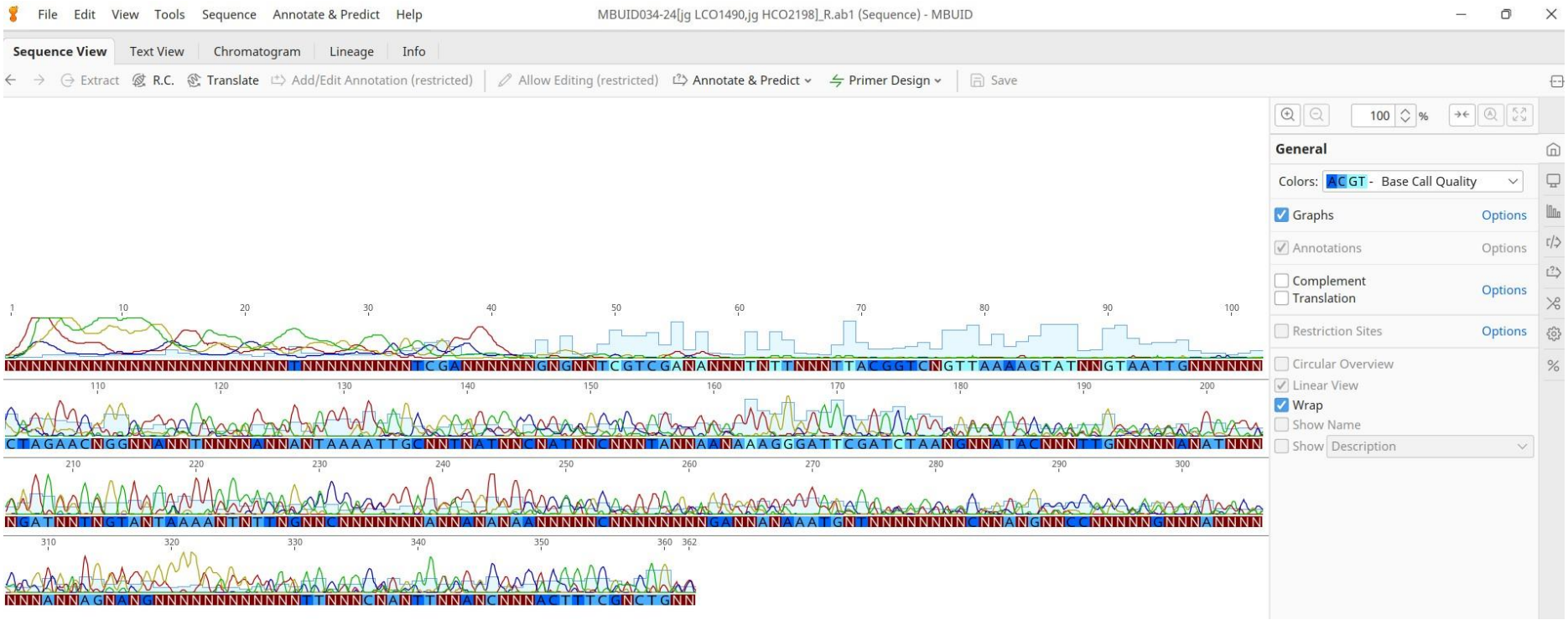
Appendix E – Low-quality DNA sequences

MBUID034 – Forward sequence



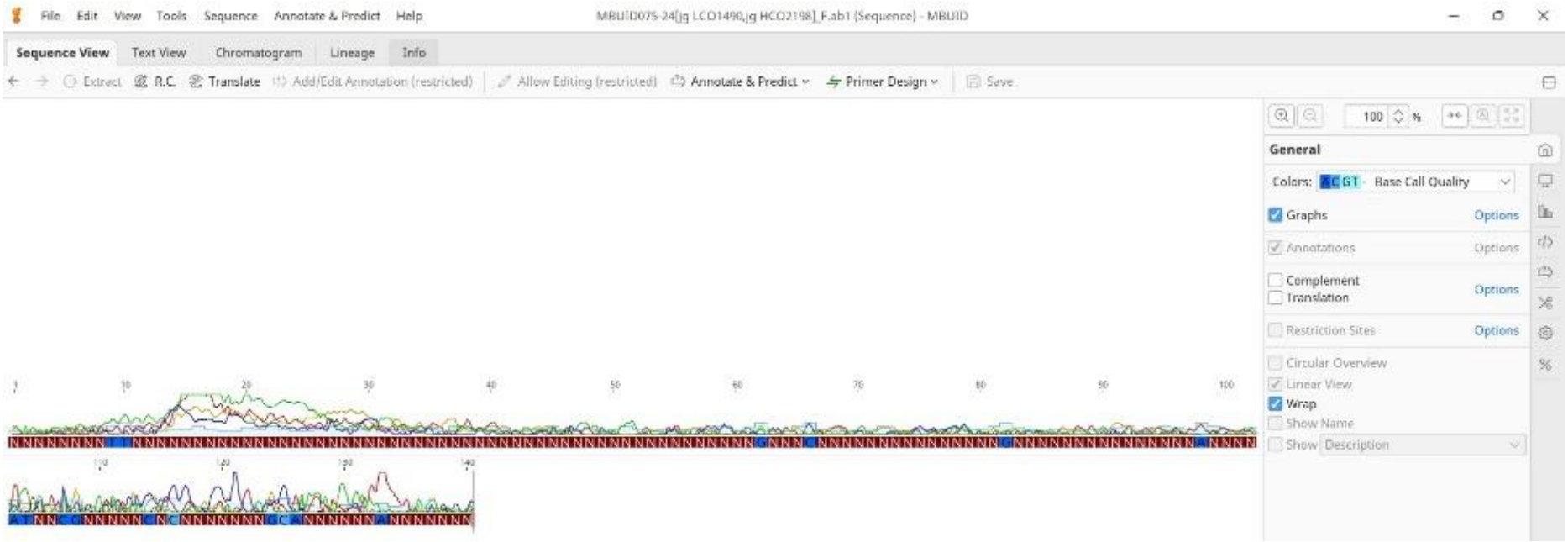
Appendix E – Low-quality DNA sequences (Continued)

MBUID034 – Reverse sequence



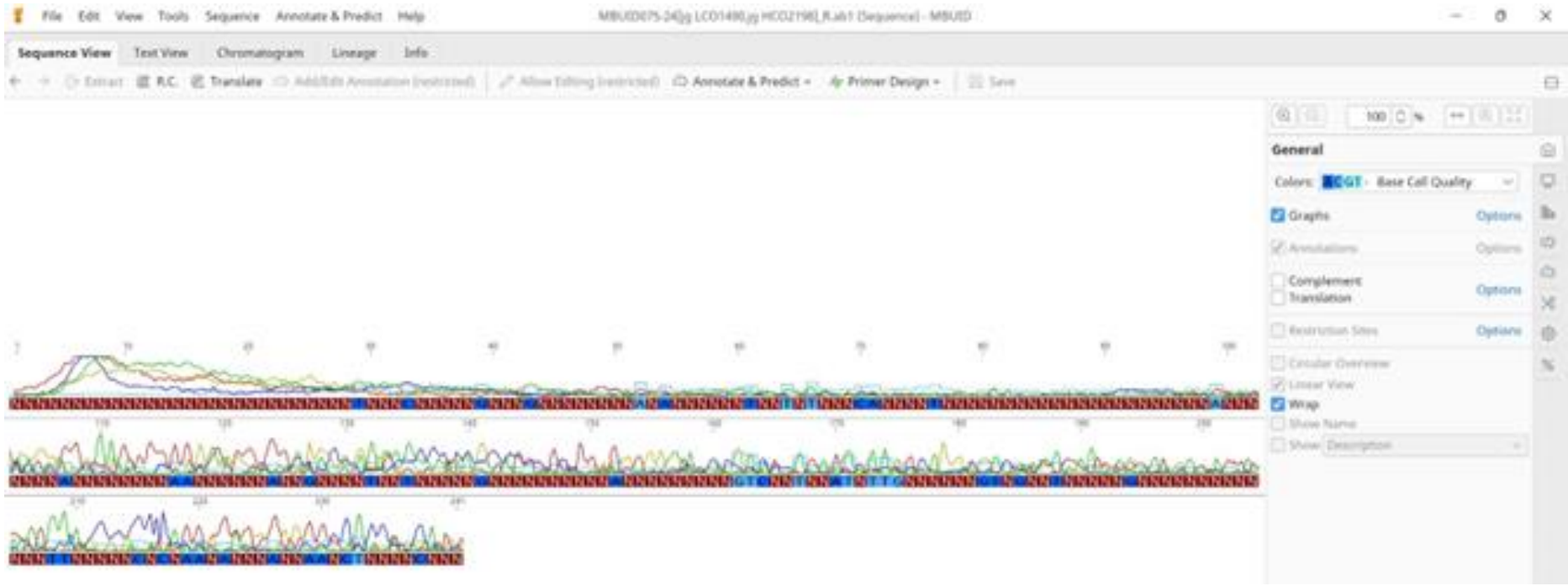
Appendix E – Low-quality DNA sequences (Continued)

MBUID075 - Forward sequence



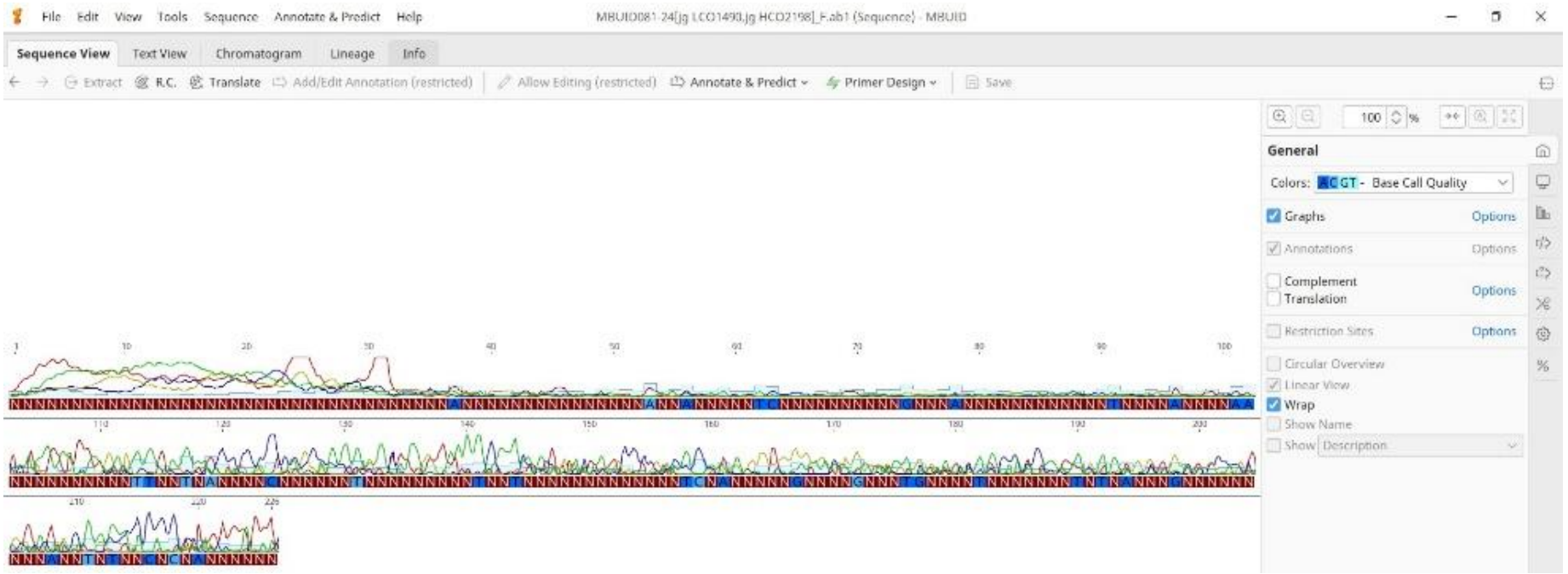
Appendix E – Low-quality DNA sequences (Continued)

MBUID075 - Reverse sequences



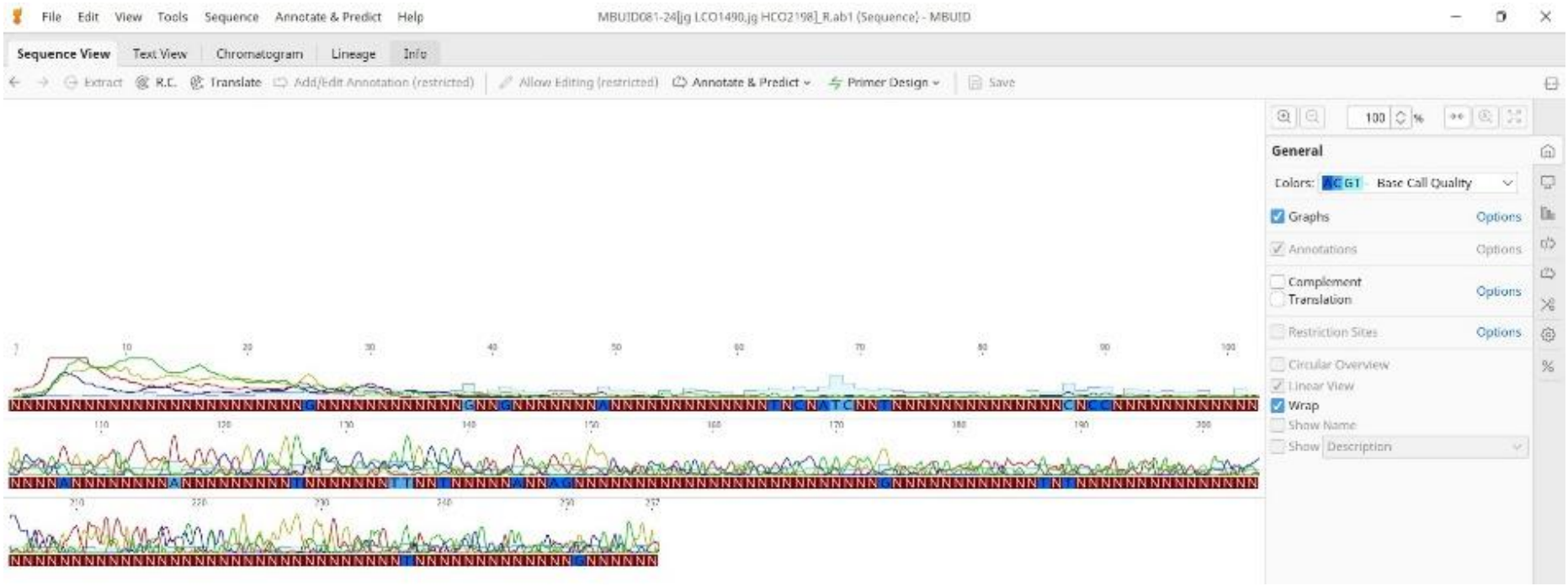
Appendix E – Low-quality DNA sequences (Continued)

MBUID081 – Forward sequence



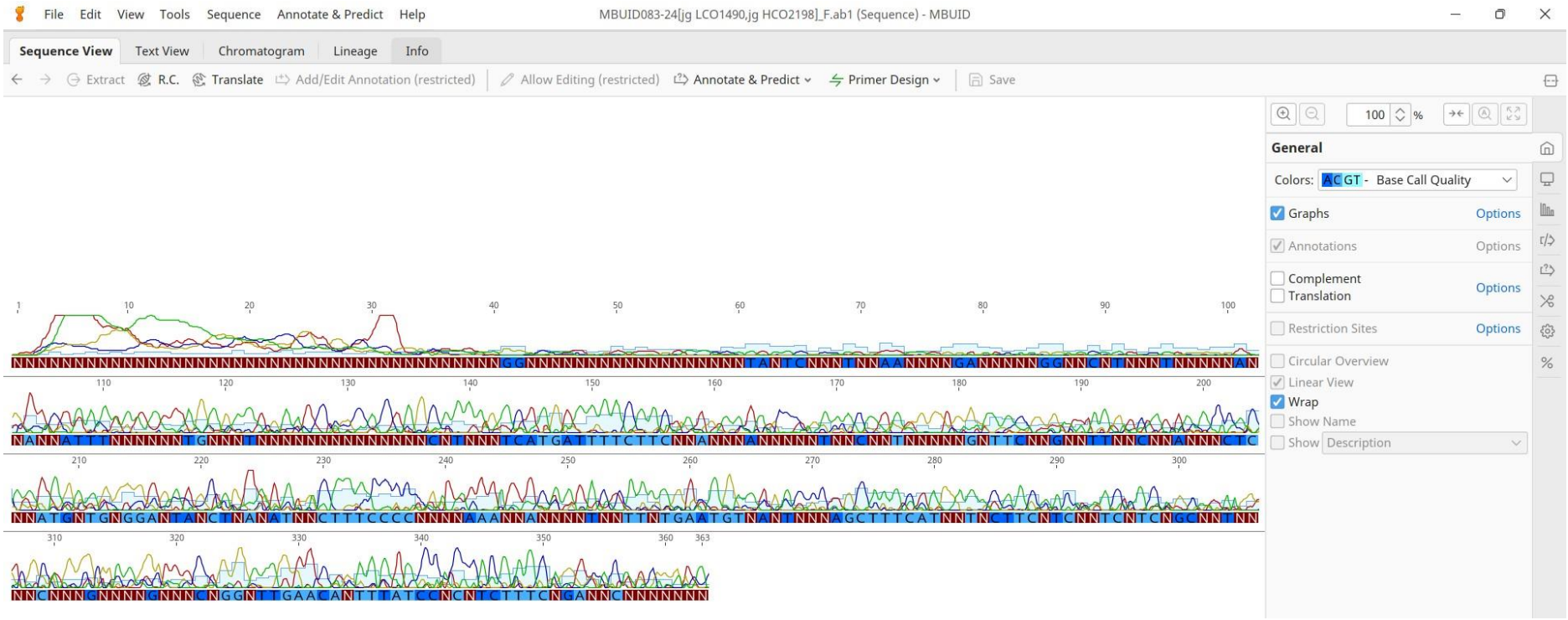
Appendix E – Low-quality DNA sequences (Continued)

MBUID081 – Reverse sequence



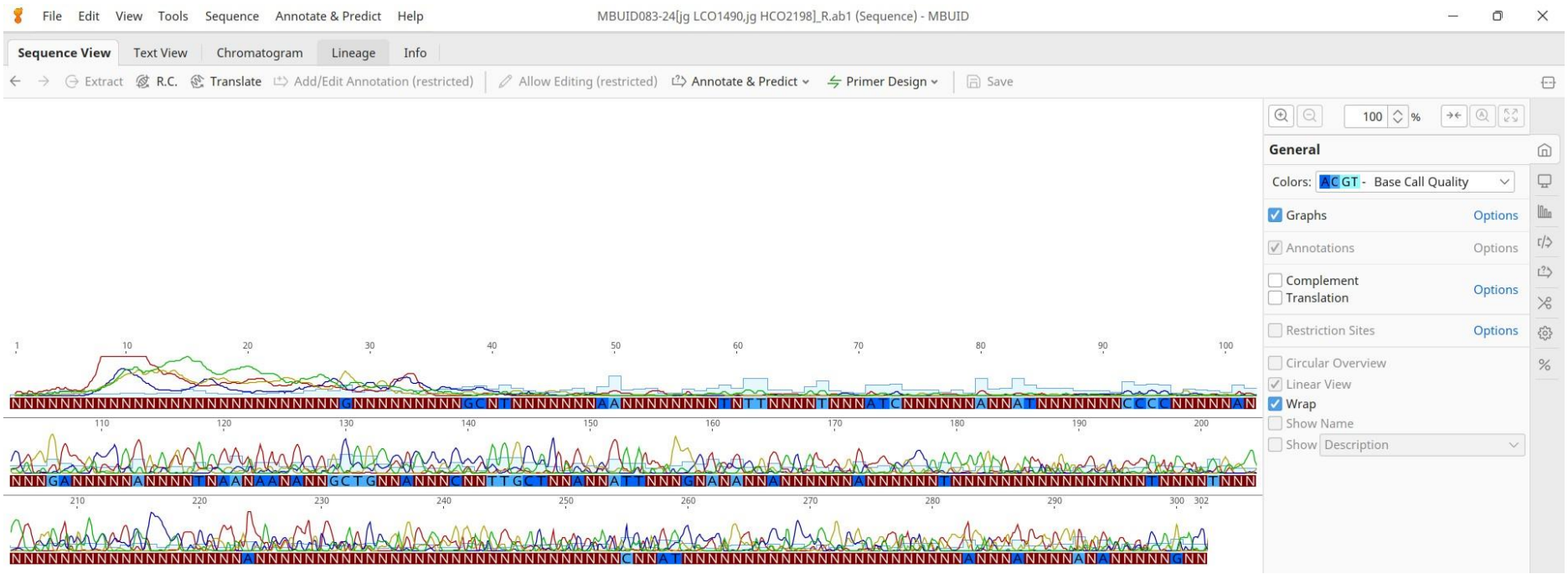
Appendix E – Low-quality DNA sequences (Continued)

MBUID083 – Forward sequence



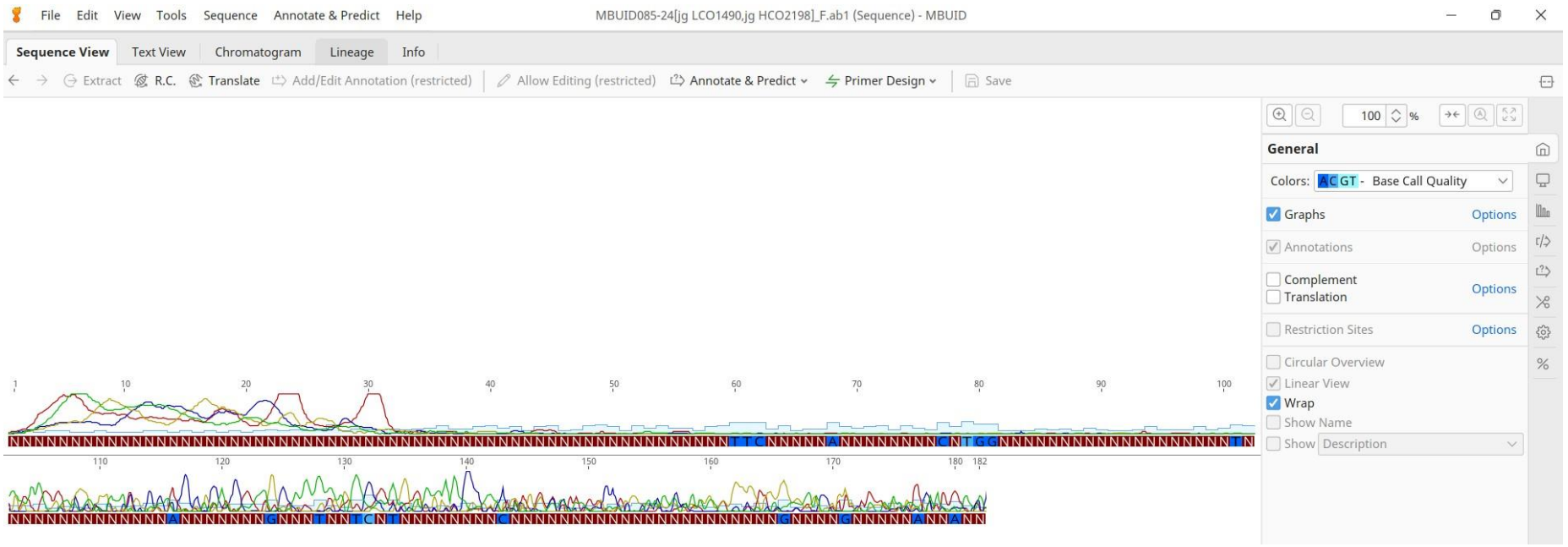
Appendix E – Low-quality DNA sequences (Continued)

MBUID083 – Reverse sequence



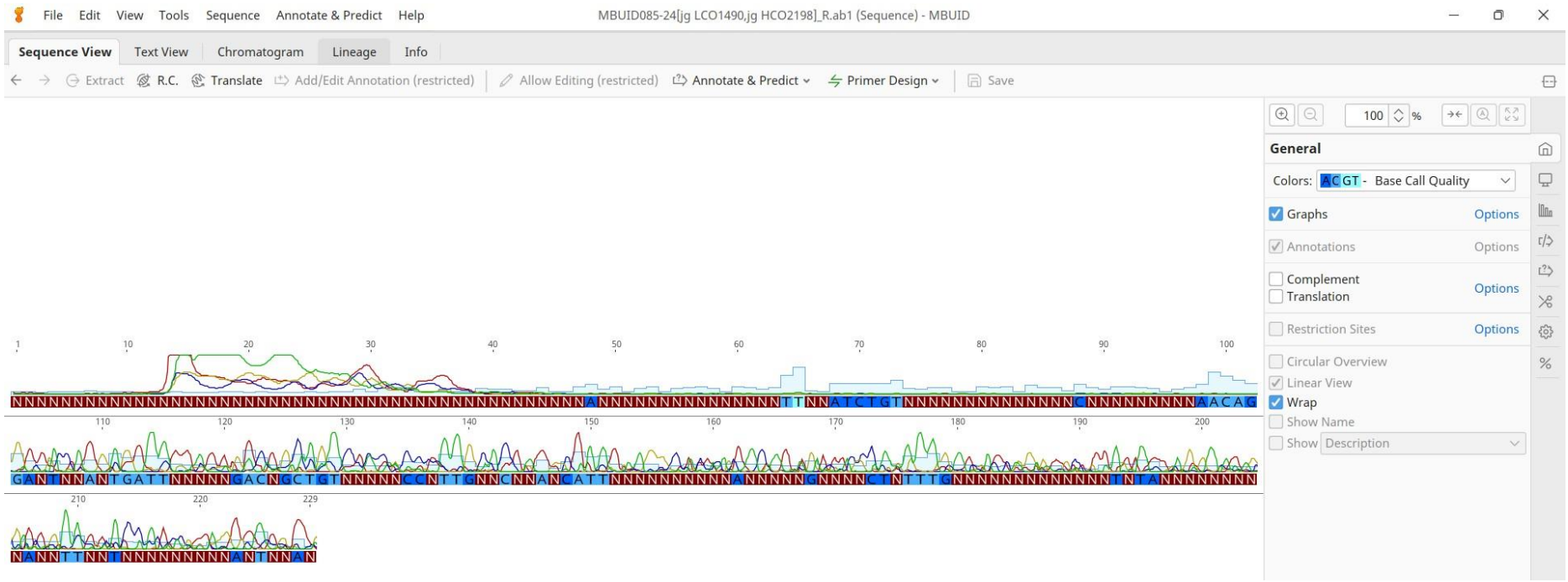
Appendix E – Low-quality DNA sequences (Continued)

MBUID085 – Forward sequence



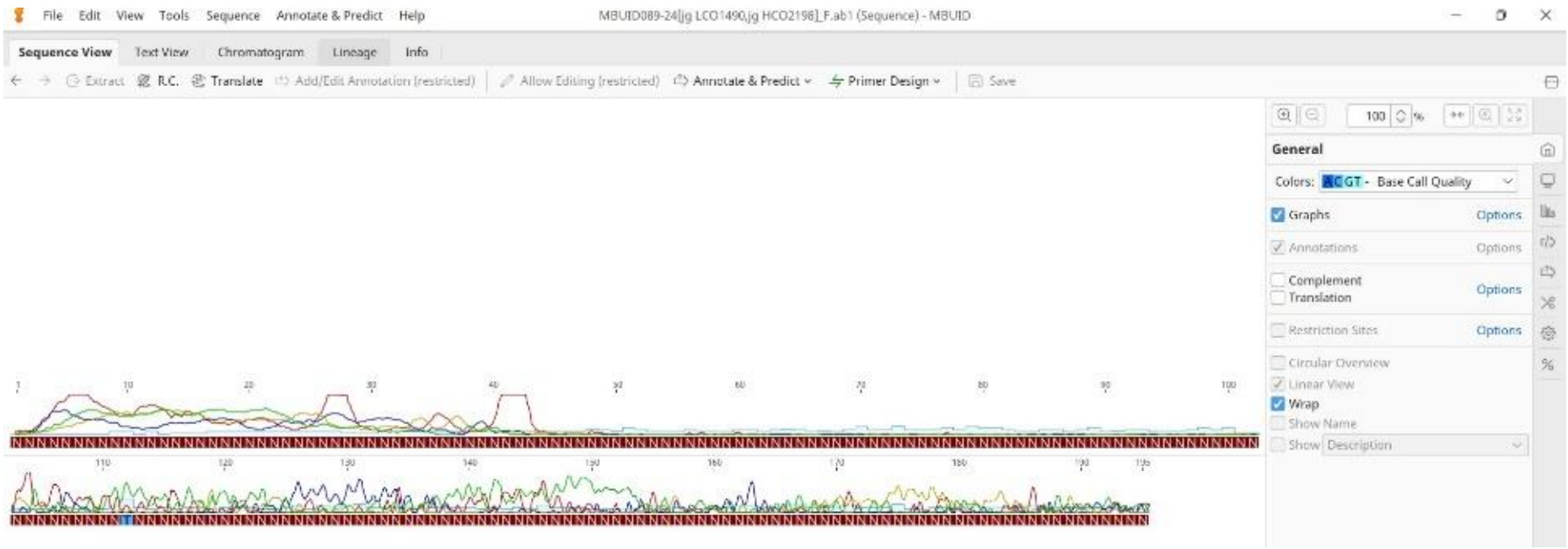
Appendix E – Low-quality DNA sequences (Continued)

MBUID085 – Reverse sequence



Appendix E – Low-quality DNA sequences (Continued)

MBUID089 – Forward sequence



Appendix E – Low-quality DNA sequences (Continued)

MBUID089 – Reverse sequence