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Phylogenetic analyses of Ascaris isolates from humans in Austria

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Abstract – English

Ascaris lumbricoides is one of the most common soil-transmitted parasitic helminths worldwide and is particularly prevalent in poor urban and rural areas with insufficient sanitation, affecting an estimated 730 million people. Chronic infestation can cause malnutrition, growth retardation, and inflammation, while severe cases lead to intestinal obstruction and even death. Austria today is considered a non-endemic region and cases diagnosed are assumed to have been imported. However, repeatedly cases have been observed in patients without travel history. Recent data indicates that *Ascaris suum*, the large roundworm of pigs, can also mature within humans. The aim of this study was to investigate the phylogenetic relationships of *Ascaris* specimens isolated from human cases in Austria. DNA was extracted from 9 individual worms isolated from different patients in Austria between 2010 and 2024. Phylogenetic analyses were based on DNA sequence dissimilarities in the mitochondrial genes cytochrome c oxidase 1 (*cox1*) and NADH dehydrogenase subunit 1 (*nad1*), and the nuclear ribosomal internal transcribed spacer region 1 (ITS1). Multiple haplotypes were identified: six for *cox1*, seven for *nad1* and six for ITS1. The Austrian isolates cluster into several groups, depending on the genetic region. The results do not allow for a clear separation between the two species *A. lumbricoides* and *A. suum*, yet rather support a recent hypothesis of a highly interbred *A. lumbricoides*/*suum* species complex, possibly including other *Ascaris* species. This study allows insight into the diversity of *Ascaris* in Austria but also highlights the complexity of its phylogeny with still several open questions.

Abstract – German

Ascaris ist weltweit einer der häufigsten parasitären Helminthen, vor allem in armen städtischen und ländlichen Gebieten mit unzureichenden Hygienebedingungen. Schätzungsweise 730 Millionen Menschen weltweit sind betroffen. Chronischer Befall kann zu Unterernährung, Wachstumsverzögerung und Entzündungen führen, schwerer Befall kann einen Darmverschluss und sogar den Tod zur Folge haben. In Österreich gilt *Ascaris* heute als nicht mehr endemisch und diagnostizierte Fälle werden als eingeschleppt eingestuft. Wiederholt auftretende Fälle bei Patientinnen und Patienten ohne Reiseanamnese stellen diese Annahme jedoch zunehmend in Frage. Jüngste Daten deuten darauf hin, dass auch *Ascaris suum*, der Spulwurm des Schweines, im Menschen heranreifen und sogar seine Geschlechtsreife erlangen kann. Ziel dieser Studie war die Analyse der phylogenetischen Beziehungen von *Ascaris* Isolaten aus humanen Fällen in Österreich. Hierfür wurde DNA aus neun Einzelexemplaren gewonnen, die zwischen 2010 und 2024 bei verschiedenen Patientinnen und Patienten isoliert wurden. Die phylogenetischen Analysen basierten auf DNA-Sequenzunterschieden in den mitochondrialen Genen Cytochrom-c-Oxidase 1 (*cox1*) und NADH-Dehydrogenase-Untereinheit 1 (*nad1*), sowie der internen transkribierten Spacer-Region 1 (ITS1) des nukleären Ribosoms. Es wurden mehrere Haplotypen identifiziert, sechs für *cox1*, sieben für *nad1* und sechs für ITS1. Die österreichischen Isolate lassen sich in mehrere Gruppen einteilen, je nach genetischer Region. Die Ergebnisse lassen keine klare Trennung zwischen den beiden Arten *A. lumbricoides* und *A. suum* zu, sondern stützen vielmehr die jüngste Hypothese eines hochgradig gekreuzten *A. lumbricoides*/*suum*-Artenkomplexes, der möglicherweise auch noch weitere *Ascaris*-Arten umfasst. Diese Studie gibt einen Einblick in die Diversität von *Ascaris* in Österreich, zeigt aber auch die Komplexität ihrer Phylogenie, die noch einige Fragen offenlässt.

1. Introduction

1.1 *Ascaris* – An overview

Soil-transmitted helminths (STH) are among the most common parasites worldwide, infesting humans and animals alike (Centers for Disease Control and Prevention [CDC], 2025). The World Health Organization (WHO) estimates that up to 1.5 billion people worldwide are infested with parasitic helminths (WHO, 2023). A meta-analysis on STH performed by Chen et al. (2024) estimates that about 643 million people were infested in 2021. Although almost all infections with helminths are technically considered infestations (Aspöck & Walochnik, 2007), the term infection is widely used in the English-language literature. Consequently, both terms are used synonymously in this work. The most common STH species are whipworms (*Trichuris trichiura*), hookworms (*Ancylostoma* spp. and *Necator* spp.) and roundworms (*Ascaris lumbricoides*) (CDC, 2025). These parasites are distributed worldwide, with the highest burdens in tropical and subtropical regions with suitable environmental conditions and limited sanitation (WHO, 2023). Despite their prevalence and their impact on public health and economy, they are considered neglected tropical diseases (NTDs) (Jourdan et al., 2018).

Among them, *A. lumbricoides* is the most prevalent intestinal nematode. Although *Ascaris* is particularly prevalent in tropical and subtropical regions (CDC, 2025), it persists in wealthier industrialized nations, albeit at significantly lower levels and mostly in connection with pig farming. Human infestations in wealthier regions were therefore classified as imported or believed to belong to the pig-specific *Ascaris suum* (Bendall et al., 2011; Nejsum et al., 2005).

1.2 Classification

The genus *Ascaris* is classified within the phylum Nematoda, order Ascaridida, superfamily Ascaridoidea and family Ascarididae (Schoch et al., 2020). This family contains several genera of major medical and veterinary importance, including *Parascaris*, *Ascaridia*, *Toxocara*, and *Heterakis*, all commonly referred to as ascarids. These nematodes share a number of biological and ecological characteristics that contribute to their pathogenic significance between humans, animals and the environment (Mugo et al., 2025).

A defining feature of ascarids is their high reproductive capacity. Adult female worms can produce thousands to hundreds of thousands of highly resistant eggs each day, which remain viable in the environment for prolonged periods of time. In addition to infesting their host's intestinal tracts, several ascarids undergo extensive larval migrations through their host's tissue, damaging it and triggering inflammatory reactions. Moreover, many ascarids show pronounced zoonotic potential. Species such as *A. lumbricoides* and *A. suum* primarily infest humans and domestic pigs, respectively, while *Parascaris* spp. parasitize equids, *Toxocara canis* and *Toxocara cati* infest dogs and cats, and *Ascaridia galli* commonly infests poultry. Importantly, these parasites are not strictly confined to their primary hosts and may also infest various other animals. And lastly, are ascarids characterized by chronic and persistent infections that disproportionately affect low- and middle-income countries, with substantial consequences for public health and local economies (Mugo et al., 2025).

1.3 *Ascaris lumbricoides* and *Ascaris suum*

Throughout human history, *Ascaris* and humans have undergone a complex coevolution, with evidence suggesting that the parasite initially infected humans before pigs and that multiple host-switching events occurred (Betson et al. 2013). As humans evolved, their parasites evolved along with them and adapted or vanished, while new parasites were obtained. Despite this complexity, it can be assumed that two significant developments in this coevolution were the settlement of humans and the domestication of animals, increasing contact between humans and animals, and their parasites (Aspöck & Walochnik, 2007; Cox, 2002). *Ascaris* is considered as one of the oldest human parasites (Betson et al. 2013) with several paleoarchaeological studies supporting the assumed long coevolution of humans and *Ascaris*, underlined by the wide distribution of the parasite. Barsch et al. (2023) detected *Ascaris* eggs in samples from a prehistoric salt mine in Hallstatt, Austria, which were dated to the Bronze Age (1158–1063 BC) and the Iron Age (750–662 BC). The authors were also able to extract DNA from these eggs, providing the earliest genetic evidence of human *Ascaris* infestation in prehistoric Europe. In Denmark, Søe et al. (2015) were able to detect *Ascaris* eggs in a Viking Age settlement (1018-1030 AD) near Viborg. The Ebers Papyrus, a medical document found in Thebes, Egypt, dating back to 1500 BC, is one of the earliest written records on intestinal nematodes (Bryan, 1930). Comparable records are known worldwide and stem from diverse eras. Based on this, and *Ascaris*' sheer size, it is

assumed that our ancestors were very well aware of this parasite (Cox, 2002; Leles et al., 2012).

In 1758, Linnaeus provided the first formal description of the human roundworm, *Ascaris lumbricoides*, followed by Goeze in 1782, who described the pig-associated species *Ascaris suum*. Despite the close relationship and morphological conservatism between these species, these classifications were recognized as valid due to their assumed host specificity, even though this assumption has been questioned over time.

In 2012, Leles et al. addressed in their study the question of whether *A. lumbricoides* and *A. suum* should be considered a single species and concluded that they should be treated as such, based on previous morphological, paleoparasitological, and genetic studies. In science, four hypotheses have emerged regarding their species classifications. The assumption of a single species, as in Leles et al. (2012), the two hypotheses that *A. lumbricoides* descended from *A. suum* (Zhou et al., 2012) or vice versa (Loreille & Bouchet, 2003), and the hypothesis that both are valid species that once diverged from a common ancestor and developed host specificity. Studies using genetic markers have yielded varying results depending on the markers used and the geographical location and sample size, which either supported the single-species hypothesis (Sadaow et al., 2018; Shao et al., 2014) or not (Dos Santos et al., 2022; Nkouayep et al., 2022; Peng et al., 2005; Søe et al., 2016). Nevertheless, host specificity has long been a main factor in classifying both species and the zoonotic potential of both species was unclear. In 2021, da Silva et al. demonstrated that *A. suum* could successfully infect five human test subjects and complete its life cycle within them, while causing similar symptoms as *A. lumbricoides*. The same was observed in other studies with *A. lumbricoides* species in pigs (Anderson, 1995; Galvin, 1968).

Despite evidence of hybridization between the two species, the occurrence of cross-contamination, and the occurrence of identical *Ascaris* genotypes in both humans and animals, the taxonomic status of the two species remains controversial and is still debated (Alves et al., 2016). How the term species should be defined in this context remains an additional controversial issue (Betson et al., 2013).

1.4 Morphology

Ascaris spp. large nematodes that range in color from yellowish to white to reddish, tapering at both ends, giving them their characteristic spindle-shaped body (Fig. 1). The

human roundworm is considered the longest parasitic nematode in the human intestine (Khan et al., 2024; Yeremiev et al., 2017). A trilabiate mouth opening is present at the anterior end of *Ascaris*, consisting of three prominent lips (Fig. 2).

The sexual dimorphism of the parasite is pronounced and allows reliable differentiation between males and females. Females are generally larger, reaching lengths of approximately 20-40 cm and an average diameter of 5 mm, whereas males are shorter, measuring about 15-20 cm and an average diameter of 3.7 mm. Males differ particularly due to the presence of a ventrolateral curled posterior end, in contrast to the females' straight, conically tapered posterior end. And finally, females are characterized by the presence of a vulva, located in the anterior third of their body, appearing as a transvers slit, whereas males possess a complexly structured cloacal region (Khan et al., 2024; Yeremiev et al., 2017).

Figure 1. Adult *Ascaris lumbricoides* specimens.

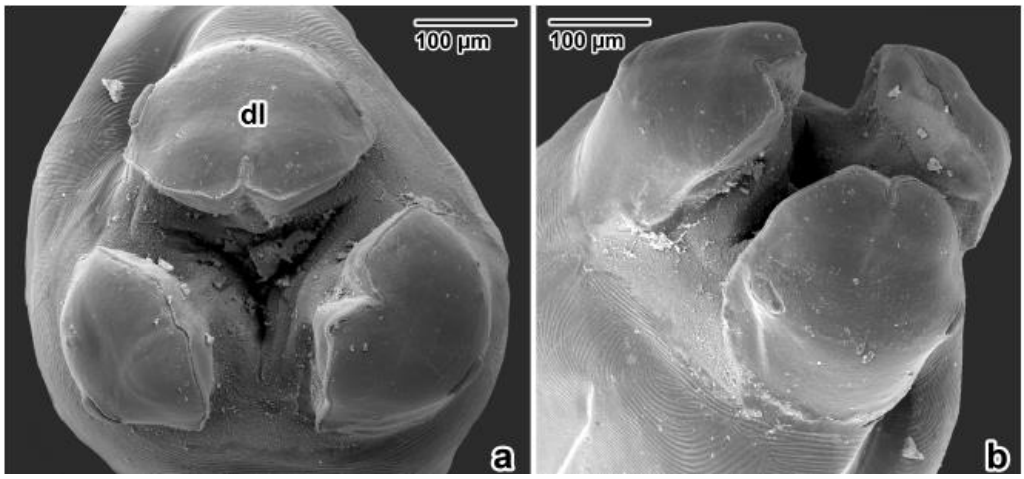

Figure 2. Trilabite mouth opening of an adult, female *A. lumbricoides*, captured via scanning electron microscope (SEM). The apical (**a**) and dorsolateral (**b**) views are shown. dl = dorsal lip (adapted from: Civáňová Křížová et al., 2023;).

Ascaris reproduces biparentally, with females producing an estimated 200,000 to 1,000,000 eggs per day (Wang & Davis, 2020; Yeremiev et al., 2017). The eggs of *Ascaris* differ in appearance depending on their fertilization status. Fertilized eggs are rounder in shape, measure approximately 40 × 60 µm, and are enclosed in a thick, multilayered shell. In contrast, unfertilized eggs possess an elongated shape, measuring 40 × 60 µm, with a thinner shell, covered with refractile granular structures (Fig. 3) (Butploy et al., 2021; Maurelli et al., 2021).

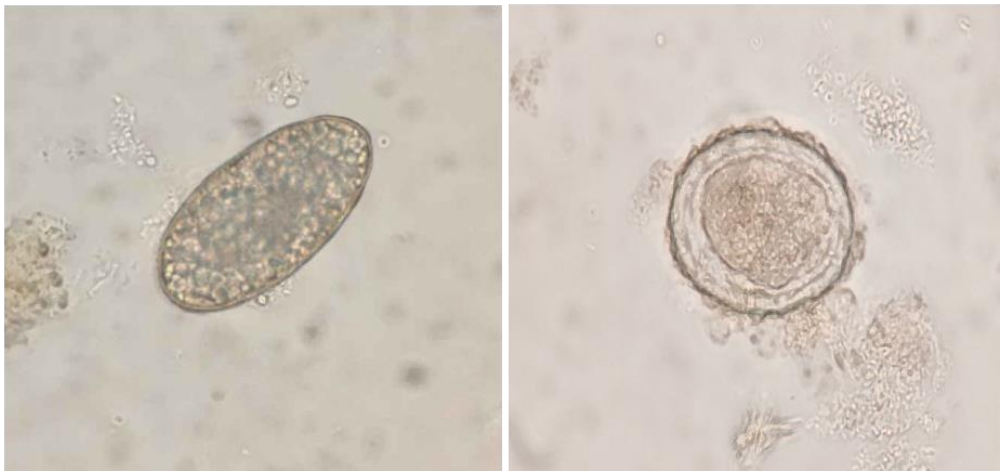

Figure 3. Microscopic images of an unfertilized *A. lumbricoides* egg (**A**) and a fertilized *A. lumbricoides* egg (**B**).

A recent scanning electron microscopy (SEM) study by Khan et al. (2024) provided detailed insights into the external morphology of *A. lumbricoides* and explored the potential for distinguishing *A. lumbricoides* from *A. suum* based on structural characteristics. In their study, they described fine morphological features, including a multilayered cuticle with distinct transverse striations, a detailed lip architecture and delicate sensory structures, such as amphids and cephalic papillae. Differences in the oral structures and variation in the cuticular surface pattern were proposed as potential species differentiation markers. Specifically, differences in oral morphology were observed, with *A. lumbricoides* showing smaller, sharper and more clearly defined lip denticles, while *A. suum* exhibited larger and blunter denticles. Despite the observations made in this study, a clear and universally applicable morphological distinction between the two species remains elusive. The study solely focused on *Ascaris* specimens from Pakistan, limiting the transferability of these

findings to other regions. Additionally, only *A. lumbricoides* specimens were examined in that study, not including directly comparable *A. suum* samples, relying instead on comparisons with previously published morphological studies. Also, it remains unclear, if the specific physiological environment within the respective host impacts morphological characteristics in any way. For instance, Sparks et al. (2015) reported measurable differences in worm size and mass between *Ascaris* populations from different geographic regions and hosts. In their study, a strong correlation between worm length and weight was observed across all samples. Worms collected from humans in Zanzibar appeared heavier than those obtained from humans in Ecuador, while pig-derived worms were lighter than the human-derived specimens. These observations suggest that morphometric characteristics may vary between *Ascaris* populations, depending on their host species and geographic location.

Consequently, although SEM can reveal subtle morphological variation and may assist in species-level differentiation under certain conditions, morphology alone seems insufficient for reliable discrimination between *A. lumbricoides* and *A. suum*. This underscores the necessity of molecular approaches in taxonomic and epidemiological research of *Ascaris*.

1.5 Epidemiology and global distribution

In 2024, the CDC estimated that approximately 772–892 million people worldwide were infested with *Ascaris*. Economically disadvantaged tropical and subtropical regions with poor sanitary conditions and inadequat sewage management are particularly affected. A global meta-analysis of *Ascaris* infections by Holland et al. from 2022 estimated a global prevalence of 11%, with the highest concentration in Melanesia (Oceania), Southeast Asia, Central America, and Central Africa (Fig. 4). Overall, their study estimates that worldwide ~730 million people are infested. Children between the age of 5 to 15 show the highest parasitic burden, while infection rate and parasite intensity decrease with age, attributed to a decline in exposure to the parasite, resulting from behavioral changes associated with adulthood (Else et al., 2020). This pattern has also been interpreted as evidence for the gradual development of a partial protective immunity against ascariasis in humans living in endemic regions, as infection intensity is often observed to decline with age in these regions (Cooper, 2002).

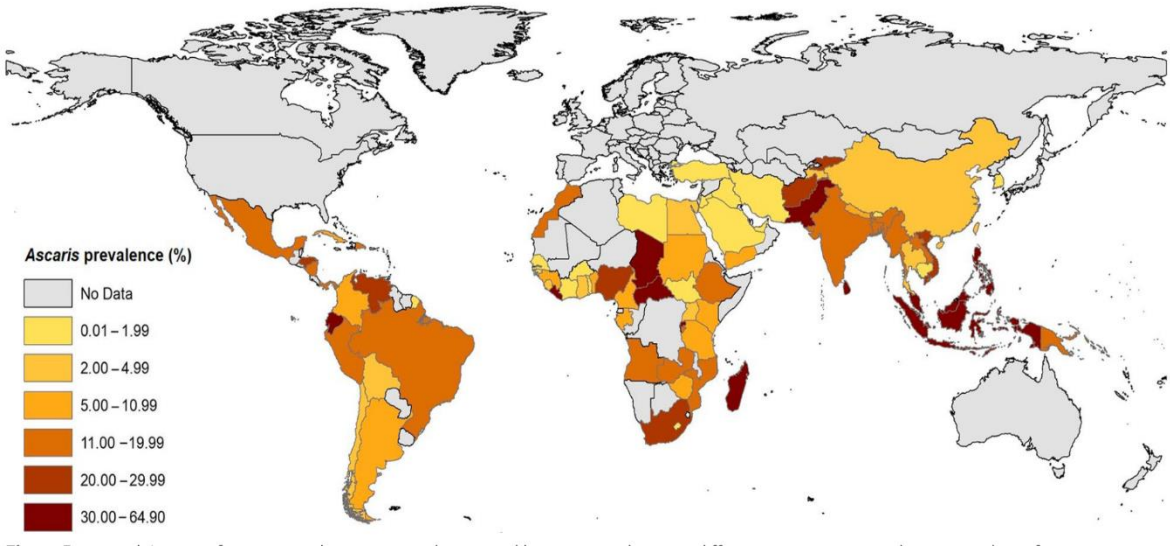

Figure 4. Estimated *Ascaris* infestation prevalence globally (Holland et al., 2022).

In industrialized regions, such as Central Europe, *A. lumbricoides* today is considered a non-endemic species due to improved sanitation and hygiene. Infections are uncommon and are typically associated with traveling to endemic regions (Fig. 4). However, studies in the UK (Bendall et al., 2011), Denmark (Nejsum et al., 2005), and the US (Chelladurai et al., 2017) confirmed the zoonotic potential of *A. suum* and raised the question of the extent to which pigs are a constant reservoir for *Ascaris* infections in these countries. Serological studies from Europe also showed high seroprevalence of *Ascaris* in the population in the Netherlands (42%), Norway (29%) and Estonia (13%) (Mughini-Gras et al., 2016; Jõgi et al., 2018; Lassen et al., 2016), indicating exposure to *Ascaris* in the local populations. Interpreting serological studies on nematodes, however, requires caution, as antibody-based assays may be affected by cross-reactions with antigens of other related helminths or environmental allergens (Jõgi et al., 2018). Despite this limitation, the findings of these studies should not be disregarded, as they indicate that exposure to *Ascaris* may be more widespread than anticipated.

1.6 Life cycle and transmission

As the life cycles of *A. lumbricoides* and *A. suum* are nearly identical (Midha, 2024), the following description will use *A. lumbricoides* as a representative example. The cycle is summarized in Figure 5.

Ascaris is a monoxenous parasite, meaning a host change is not required. Infection occurs directly through fecal-oral ingestion of embryonated and infectious eggs via contaminated water or food. Activated by the environmental changes inside the host, caused by carbon dioxide, sodium bicarbonate and mechanical stimulation (Mkandawire et al., 2022), the L3 larva hatches in the large intestine, where it is initially still surrounded by the L2 cuticle. In the caecum and proximal colon, the larva penetrates the mucous membrane of the large intestine and reaches the liver via the portal vein, where it sheds the L2 cuticle. After molting and a growth phase, the L3 larva exits the liver and traverses the blood vessel system via the heart to the lung tissue. There, the larva penetrates the alveoli and migrates towards the pharynx, where it is expectorated and subsequently swallowed once more, where it reaches its final destination: the small intestine. There the L3 larva undergoes a molt into the L4 stage, and subsequently into its adult reproductive stage. The adult worms reside in the lumen of the small intestine with a lifespan of approximately one to two years (Else et al., 2020). The period between ingestion of an infectious egg and complete development of the parasite is estimated at 2-3 months (CDC, 2019). The worms mate in the small intestine and shed eggs that are excreted with the hosts feces. Freshly passed eggs are considered non-infectious and require a period of time in the external environment to develop and become infective. Embryonation proceeds under suitable environmental conditions, with temperature and humidity serving as key factors (Wang & Davis, 2020). At approximately 30 °C, eggs typically reach the infective stage within 10-14 days, whereas cooler conditions, such as 17 °C, can extend the maturation period to 45-55 days. Failing to embryonate leads to non-infectiveness of the eggs. Due to their highly durable shell, *Ascaris* eggs show high environmental resilience, remaining infectious for extended periods of time, including estimations ranging to 6 years and even up to 14 years (Else et al., 2020). This favors reinfections in endemic regions, despite regular mass treatment of the local population (Wang & Davis, 2020).

However, the life cycle of *Ascaris* is complex, and the exact processes and routes of infection are not yet fully discovered or understood (Else et al., 2020). For instance, ingestion of infectious eggs is arguably the most prevalent route of infection, but it is not the sole one.

Ishiwata et al. (2004), as well as Schneider & Auer (2016) named the ingestion of larvae through the consumption of raw meat, like liver, as an additional route of infection. Why

the life cycle of *Ascaris* involves migration from the liver to the lung, thereby exposing itself to higher risks, also remains questionable. This migration is assumed to be beneficial for the parasite's fitness, leading, for example, to increased growth, yet the question remains unresolved (Else et al., 2020).

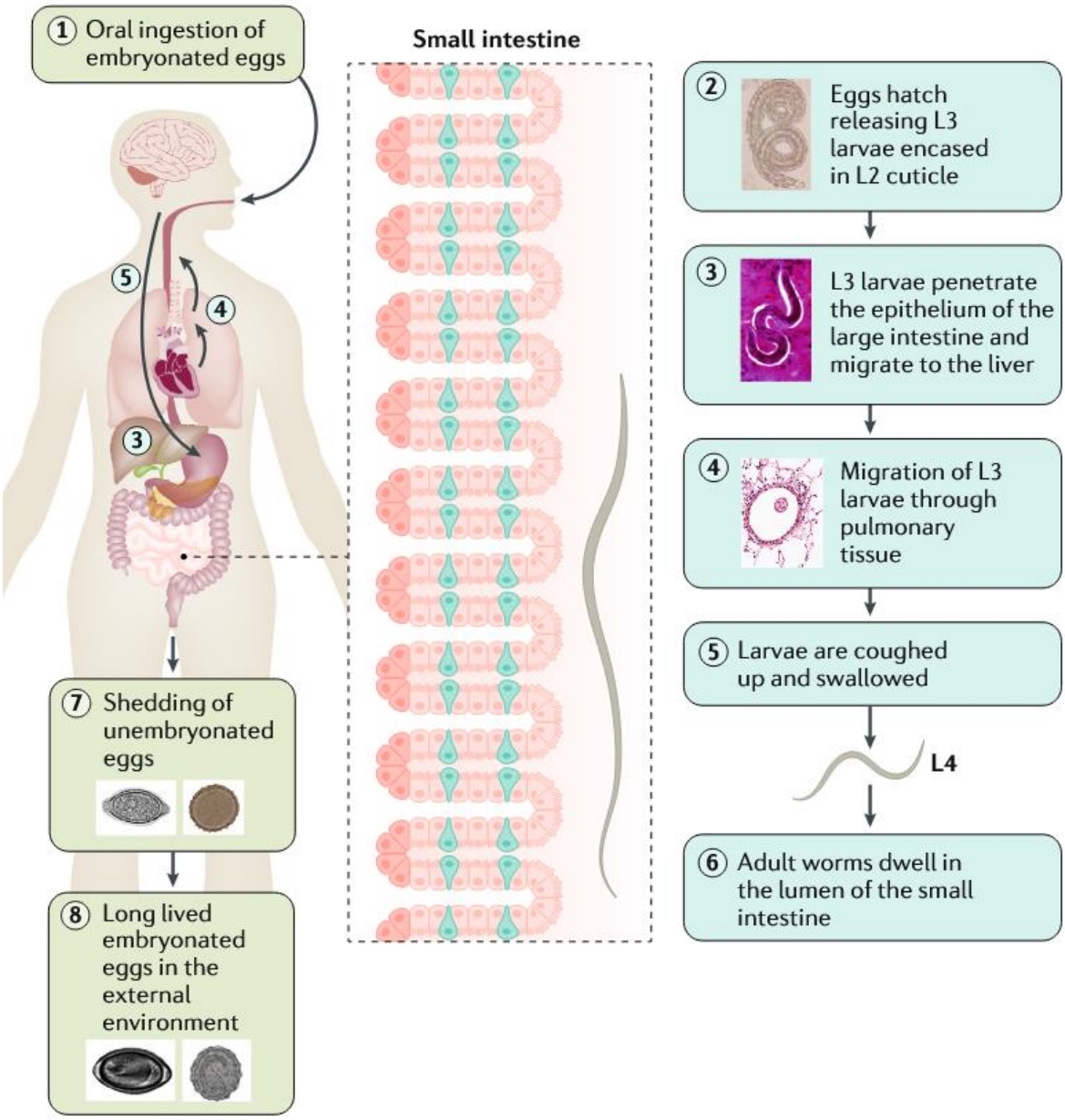

Figure 5. Life cycle of *A. lumbricoides*, from the ingestion of infectious eggs (**1**) to their excretion and development in the environment (**8**) (adapted from: Else et al., 2020).

1.7 Disease in humans

Ascaris infection and the various clinical pictures associated with it are summarized under the collective term “ascariasis”. A mild infection usually progresses without symptoms in

healthy individuals, with morbidity manifesting in only 8-15% of ascariasis patients (Dold & Holland, 2011). When the infection becomes more severe, intestinal problems and complications such as diarrhea and abdominal pain occur. Ascariasis is considered a prime example of a chronic disease that particularly affects the growth of children. Severe infections lead to nausea, vomiting, malabsorption, and digestive problems, meaning that food intake is reduced and important nutrients cannot be absorbed, accumulating to vitamin deficiency (Else et al., 2020; Jourdan et al., 2016; Sparks et al., 2015; Strunz et al., 2016). Although not yet fully understood, cognitive impairment is linked to heavy *Ascaris* infection, presumably through the malnutritional effects or through the systemic low-grade inflammatory reaction caused by larval migration (Else et al., 2020). In particularly severe cases, a high worm burden can cause partial or complete obstruction of the small intestine (Fig. 6) or, following migration into the biliary or pancreatic ducts, obstruction of these ducts, resulting in secondary diseases such as cholangitis, cholecystitis, pancreatitis, and other inflammatory diseases. Consequently, although uncommon, intestinal rupture can occur, which can be fatal if left untreated (Jourdan et al., 2018; Midha, 2024; WHO, 2023).

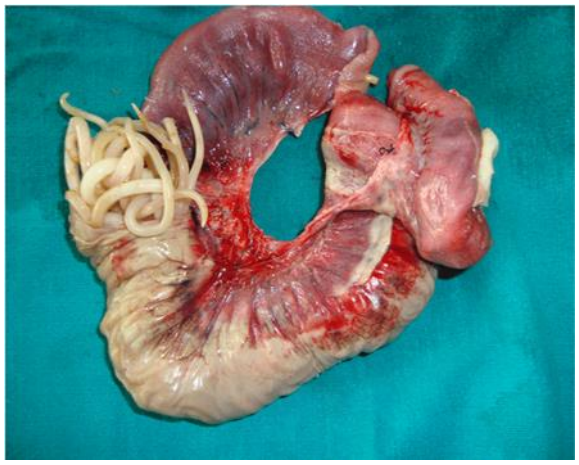

Figure 6. Resected ileum teeming with numerous roundworms (Gazula, 2016).

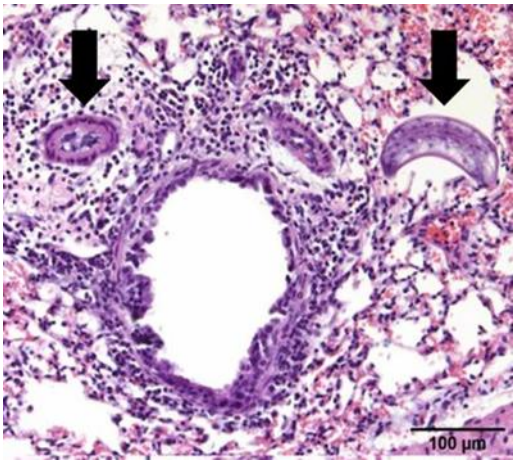

Figure 7. Histological image of lung tissue showing representative bronchovascular bundles following *Ascaris* lung infection (Weatherhead et al., 2018).

Acute symptoms most commonly occur during the migration phase of the larvae from the intestine to the lungs, commonly referred to as visceral larva migrans (VLM) syndrome (Midha, 2024; Schneider et al. 2015). The tissue damaged by migration activates the body's immune response to the parasite, accompanied by inflammatory reactions (Midha, 2024).

In cases of high worm loads, bleeding of the mucous membrane of the gastrointestinal tract can lead to anemia (Jourdan et al., 2018). If larvae become entrapped in the liver during migration, they trigger the formation of “milk spots”, which are white, fibrotic lesions resulting from localized immune-mediated encapsulation of the parasites (Pérez et al., 2001). Upon reaching the lungs (Fig. 7), the larvae may induce eosinophilic pneumonitis, an inflammation of the lung tissue, commonly referred to as Loeffler’s syndrome. Clinical manifestations can include shortness of breath, coughing, haemoptysis, chest pain and fever, with severe cases potentially progressing to pleurisy or pleural effusion (Dold & Holland, 2011; Hoenigl et al., 2010; Jourdan et al., 2018). The body's immune system responds to parasitic infections of the lungs with a local type 2 immune response. This response is intended to provide protection against the infection but can manifest itself pathologically, resulting in long-lasting immunological hypersensitivity of the respiratory tract, similar to asthmatic diseases (Jourdan et al., 2018; Weatherhead et al., 2018). Consequently, Jõgi et al. (2018) were able to associate *Ascaris* infection with reduced lung function, as well as an increased risk of asthma in human males.

Although the majority of infections are asymptomatic or only lead to mild symptoms, it is estimated that between 20,000 and 60,000 people die each year from severe *Ascaris* infections (Nkouayep et al., 2022).

1.8 Diagnosis and treatment

The diagnostic approach to human ascariasis is largely determined by the clinical presentation, the stage of infection, as well as the local epidemiological context. In non-endemic regions, where infections with *Ascaris* are rare and often mild or asymptomatic, cases are frequently identified incidentally, typically when adult worms are expelled in the stool (CDC, 2019), as also observed in the majority of Austrian cases. When clinical symptoms indicate visceral larva migrans (VLM) syndrome or Loeffler’s syndrome, a chest radiography may reveal signs of parasitic infiltration of the lung, which can subsequently be confirmed through serological testing (CDC, 2019; Jourdan et al., 2018; Schneider et al., 2015).

In a global context, direct microscopic detection of *Ascaris* eggs in stool samples remains the standard method for detecting infection. The methodology is considered cost-effective, relatively easy to perform, requires comparatively little equipment, and the characteristic

physiology of the eggs allows for clear differentiation from other helminth species, making it ideal for large-scale use in endemic regions (CDC, 2019; Holland et al., 2022; Jourdan et al., 2018). Additionally, the quantity of detected eggs provides an indication of the worm burden present in the host. The standard quantitative microscopic method is the Kato-Katz thick smear method recommended by the WHO; although, alternative methods such as formalin-ether concentration, McMaster, FLOTAC, and Mini-FLOTAC can be employed as well. Despite its widespread use, microscopic examination presents several important limitations. Low infections may go undetected, and microscopic detection of eggs is impossible before worms mature and begin producing eggs, which can take several weeks after the initial infection. Moreover, egg output may vary considerably between days and among stool samples, potentially leading to inaccurate estimations of infection intensity. Additionally, larval migration through organs, such as the lungs or liver, cannot be detected through stool microscopy, since they do not produce eggs in the migration stage (Else et al., 2020; Jourdan et al., 2018; Vlaminck et al., 2014). Alternatively, larvae can be diagnosed during pulmonary migration in the patient's sputum (CDC, 2019) or adult worms through targeted drug-induced excretion. However, these methods are more complex and expensive and are rarely used for primary identification. In addition, the morphological similarity between *A. lumbricoides* and *A. suum* prevents species specific differentiation (Jourdan et al., 2018).

Due to the limitations of microscopic diagnostics, serological methods such as enzyme-linked immunosorbent assays (ELISA) for the detection of anti-*Ascaris* antibodies are considered a valid alternative, as these methods can provide positive results as soon as extraintestinal migration of the larvae has started. However, these assays also face limitations: necessary reagents and materials are not commercially available everywhere and adequate infrastructure for storage and cooling is not feasible in all settings. Additionally, they can produce cross-reactions with antibodies against other helminths, possess limited species-specificity, and cannot reliably distinguish between active and past infections (Auer, 2021; Jourdan et al., 2018).

In the recent past, molecular methods like polymerase chain reaction (PCR) and quantitative real-time PCR (qPCR) have become valuable research and diagnostic tools. They enable detection of DNA with high sensitivity and specificity, even in samples with low parasite burdens or degraded material, making it ideal e.g. for paleoparasitological studies. These assays also allow strain differentiation, enabling epidemiological studies on

zoonotic transmission (Vlaminck et al., 2014). Despite these advantages, molecular tools are, like serological methods, technically demanding and costly, requiring specialized laboratory equipment. Their use is therefore often limited to reference laboratories and research settings and is typically not feasible as routine diagnostics in economically disadvantaged regions or in the field (Else et al., 2020).

The treatment of ascariasis, while generally effective, still presents certain challenges. Benzimidazole derivatives, such as albendazole and mebendazole, as well as diethylcarbamazine (DEC), are potent anthelmintic agents, yet their therapeutic efficacy may vary, compared to treatment of other helminth infections. Albendazole is currently regarded as the drug of choice by the WHO, which recommends a single oral dose of albendazole (400 mg) or mebendazole (500 mg) as standard treatment (WHO, 2023). The CDC also recommends a single oral dose of ivermectin (150-200 µg/kg) for treatment (CDC, 2024). Patients suffering intestinal obstruction should be given fluids and electrolytes intravenously, as well as antibiotics, if systemic infection is suspected. After treatment with anthelmintics, worms can be removed endoscopically or surgically (Fig. 6), depending on severity (Jourdan et al., 2018).

1.9 Molecular epidemiology and phylogeny of *Ascaris*

The rise of molecular epidemiology and molecular phylogeny revolutionized parasitology, expanding our scientific understanding of parasite transmission routes, population structure and evolutionary relationships. The integration of genetic data allows infections to be examined on a global scale and between populations with a level of resolution that cannot be achieved through microscopy, serology or routine surveillance alone (Hide, 2025; Lymbery & Thompson, 2012).

1.9.1 Molecular epidemiology

Epidemiology is the scientific study of determination of diseases, as well as the dynamics of their transmission within and across populations. In parasitology, it focuses on how parasites are transmitted between hosts and between host populations, and the biological and health-related consequences of such infections. Both sciences followed a strongly empiricist approach in their development, drawing conclusions about patterns and risk

factors based on the frequency and prevalence of diseases or infections (Lymbery & Thompson, 2012).

Molecular epidemiology utilizes molecular biological techniques to investigate the distribution and determinants of infections across populations, with the ability to detect and characterize genetic variability within. This development greatly expanded the scope of parasitological research by enabling the reconstruction of parasite evolutionary relationships, the identification of previously unrecognized taxa, the resolution of taxonomic uncertainties and the analysis of genetic diversity within parasite populations. Furthermore, it allows the integration of parasite genotypes with additional data of host, geography and environment, the reconstruction of transmission pathways, the identification of sources of infection and the evaluation of connections between parasite populations (Hide, 2025; Lymbery & Thompson, 2012). The significance of molecular epidemiology has become particularly evident in the context of mass drug administration (MDA) programs. The increase in such programs has, for instance, led to a decline in helminth infections in the affected areas. However, the evolutionary pressure put on parasite populations by these programs can result in the emergence and spread of drug-resistant strains, compromising the efficacy of these measures. The emergence of such developments can be detected and monitored through molecular surveillance (Landeryou et al., 2025).

The application of molecular epidemiology in *Ascaris* research stems to some degree from the limited discriminatory power of morphological characteristics to separate *A. lumbricoides* and *A. suum* (Liu et al., 2012). Conventional diagnostic methods can confirm an infection, but do not allow taxa to be determined, nor do they provide any information about whether a zoonotic infection occurred and whether cases were locally acquired or imported. Molecular approaches can overcome some of these limitations, but do not eliminate them entirely (Betson et al., 2014; Liu et al., 2012).

1.9.2 Molecular phylogeny and haplotype analysis

Molecular phylogeny is closely linked to molecular epidemiology, as it uses DNA or protein sequence data to reconstruct evolutionary relationships between parasite lineages. Haplotype analysis represents a practical application of molecular phylogenetic principles at the population level (Horiike, 2016).

A haplotype is a group of genomic variations that are usually inherited together, whereby a specific haplotype refers to a particular combination of sequence variation. A haplotype can hereby differ in one or more nucleotides (National Human Genome Research Institute [NHGRI], 2026). The haplotype network is a visualization tool that can graphically represent the relationship between different haplotypes in a population or species. Position and grouping of haplotypes in the network can help identify genetic patterns that offer insight into hybridization processes, mutational steps or geographical distribution of certain haplotypes (Davinack, 2024).

Mitochondrial markers cytochrome C oxidase 1 (*cox1*) and NADH dehydrogenase subunit 1 (*nad1*), and ribosomal marker internal transcribed spacer 1 (ITS1) have been the most used DNA markers in *Ascaris* research, besides microsatellite markers (Cavallero et al., 2013; dos Santos et al., 2022; Sadaow et al., 2018). Ribosomal DNA, like ITS1, have been used in the past to distinguish between closely related or morphologically similar species (Ishiwata et al., 2004), due to their high degree of sequence variation. Mitochondrial targets, such as *cox1* and *nad1*, have been used more frequently in studies that aimed to analyze phylogenetic relationships (Peng et al., 2005; Nejsum et al., 2017). The frequent use of these markers rendered the works of Ishiwata et al. (2004) and Peng et al. (2005) two of the most frequently cited works in *Ascaris* research. Although no definitive marker for species differentiation has been found to date, combination of markers has proven useful in research (Betson et al., 2014).

1.10 *Ascaris* infections in Austria

The current incidence of human ascariasis in Austria is not well documented, as systematic surveillance data is lacking and the diagnosis is not reported in Austria. However, earlier studies indicate a decline in helminthic infestations over the course of the 20th century. Tomaso et al. (2001) analyzed 142,426 stool samples collected in Tyrol between 1990 and 2000 and reported a decrease in helminth infestations overall. Based on datasets of the federal public health laboratory in Innsbruck, they determined that approximately 26% of the 582 examined samples between 1945 and 1949 were positive for intestinal helminths. By 1985, the overall positivity rate had declined to 0.98%, and between 1990 and 2000 only 336 samples (0.24%) were tested positive for helminths. Among these samples, 43 (12.8%) were positive for *A. lumbricoides.*

Tomaso et al. (2001) suggested that most human ascariasis cases in Austria were likely acquired through oral uptake fecally contaminated food abroad; however, they also reported instances in Tyrolean patients who had no history of travel. More recent studies provide evidence that autochthonous ascariasis continues to occur in Austria despite the extremely low level of endemic transmission. These include two documented cases of pulmonary ascariasis in Austrian patients reported by Hoenigl et al. (2010) and a serological survey by Schneider and Auer (2016), which showed that 13.2% of 4,481 serum samples collected between 2012 and 2014 were tested positive for anti-*Ascaris* antibodies. In both studies, the infections were assumed to be caused by *A. suum*. Auer (2021) estimates that, although only a small number of cases are documented, several hundred patients per year suffer from ascariasis. This is supported by the fact that 12% of serum samples analyzed in recent years from patients suspected of having visceral larva migrans syndrome were tested positive for antibodies against *A. suum*. However, this estimation is limited by the fact that only a small number of physicians requested serological testing.

Regarding a potential reservoir, pigs in Austria are confirmed hosts of *A. suum*, bearing zoonotic potential. A study conducted by Joachim et al. (2021) demonstrated a high prevalence of infestation on Austrian swine farms. On 15 of 18 farms examined, 19% of livers inspected exhibited “milk spots” at slaughter, indicative of *Ascaris* larval migration. In addition, eggs have been detected in 13% of fecal samples. Serological screenings further revealed a positivity rate of 69% among the sampled pigs. It is estimated that one-third of domestic farms raising pigs are infected with *Ascaris* (Auer, 2021). This highlights the potential of pigs as reservoir for *Ascaris* in Austria and the zoonotic risk associated with it, especially through the agricultural use of untreated pig manure, which can lead to environmental contamination of crops and soil.

However, several systemic weaknesses hamper the generation of accurate data on *Ascaris* in Austria, making it difficult to monitor the local situation. Firstly, there is no mandatory reporting obligation for ascariasis in Austria, or Europe in general, meaning that many cases go unrecorded and epidemiological development cannot be tracked accurately. Secondly, due to the rarity and widespread unfamiliarity of the disease, doctors treating patients lack awareness of it (Auer, 2021). There are no pathognomonic symptoms and the disease is difficult to differentiate from other diseases, especially in the absence of a noticeable travel history. This can result in cases not being diagnosed.

Given this background, the epidemiological situation in Austria is not entirely clear. The assumed occurrence of autochthonous cases, the potential reservoir in the domestic pig population and the limited epidemiological surveillance due to the lack of data, raise the question if there is local transmission of *Ascaris*. The controversial taxonomic situation between *A. lumbricoides* and *A. suum* further complicates the matter and highlights the need for further studies, within Austria, but also Europe.

1.11 Aims of this study

Although Austria today is considered a non-endemic region for *Ascaris lumbricoides* and cases diagnosed in humans are assumed to be imported, infections have repeatedly been detected in individuals without travel history. Occasional autochthonous infections have thus been assumed, but no molecular data on *Ascaris* isolates from Austrian patients are available.

Therefore, the aim of this study was to perform detailed phylogenetic analyses of *Ascaris* isolates obtained from human cases in Austria over the past 14 years and to genetically compare these isolates with reference *Ascaris* sequences from around the world available in the GenBank database. To ensure comparability, three genetic loci were targeted: the mitochondrial genes cytochrome c oxidase subunit 1 (*cox1*) and NADH dehydrogenase subunit 1 (*nad1*), as well as the nuclear ribosomal internal transcribed spacer 1 (ITS1) region.

This study offers insights into the genetic diversity of *Ascaris* in a European country, addressing a research gap, since studies to date have focused largely on endemic countries, while having been neglected in regions considered “non-endemic”. The significance of this work lies in its potential to provide information about possible sources of infection, the accuracy of the traditional picture of transmission routes, and to understand if changes in transmission routes, dynamics or host-specificities have occurred.

2. Materials and methods

2.1 Study site and sample selection

The study was conducted at the Institute for Specific Prophylaxis and Tropical Medicine at the Center for Pathophysiology, Infectiology, and Immunology of the Medical University of Vienna.

The sample material consisted of archived *Ascaris* worms isolated from human samples sent to the institute between 2010 and 2024 for laboratory diagnostics. All samples had been sent in from medical facilities in Austria; no personal patient data was included in the study. Samples from patients with a known history of travel were noted as such (Tab. 1).

Table 1. Samples used in this study. 9 samples were available. The date of sample isolation and known travel history of the patients are given. Samples with missing travel data were marked with "no data", otherwise indicating that no travel took place or the countries visited by the patients.

Sample name	Year of isolation	Travel History
10A1	2010	no data
10A2	2010	no data
16A1	2016	Indonesia, Singapore, Thailand
18A1	2018	no data
18A2	2018	no travel within several years
19A1	2019	Indonesia
23A1	2023	no data
24A1	2024	Bulgaria
24A2	2024	no data

2.2 Primer selection and design

To ensure comparability and to enhance sensitivity, target regions and primers from established and frequently cited studies were used. Following literature research, the primers from Peng et al. (2005) were selected for cytochrome c oxidase 1 (*cox1*) and NADH dehydrogenase subunit 1 (*nad1*) (Tab. 1), those from Ishiwata et al. (2004) for ITS1 amplification and sequencing. Since not all samples could be successfully sequenced with the established ITS1 primers, a new set of primers was developed and additionally used during the study (Tab. 2). New primers were designed using OligoCalc (http://oligocalc.eu/) and checked for sources of error. Attention was paid to the length of the primers, the GC content, melting temperature, and the possible formation of secondary structures. All primers were ordered from Microsynth AG (https://www.microsynth.com/).

Table 2. Forward and reverse primers used in this study, including new ITS1 primers As NF-ITS1 and As NR-ITS1 designed during this study.

Locus	Primer	Primer name	Primer sequence (5‘ - 3‘)	Annealing Temp.	Amplicon size	Source
cox1	Forward	As-Co1F	TTTTTTGGTCATCCTGAGGTTTAT	55°C	~ 400 bp	Peng et al. (2005)
	Reverse	As-Co1R	ACATAATGAAAATGACTAACAAC			
nad1	Forward	MH5F	TATGAGCGTCATTTATTGGG	55°C	~ 380 bp	Peng et al. (2005)
	Reverse	As-NDR	GCATCAAATAGCCAACAAATAC			
ITS1	Forward	F2662	GGCAAAAGTCGTAACAAGGT	52°C	~ 460 bp	Ishiwata et al. (2004)
	Reverse	R3214	CTGCAATTCGCACTATTTATCG			
	Forward	As NF-ITS1	CGATGAAAGGTGGAGAGAAA	52°C	~ 460 bp	This study
	Reverse	As NR-ITS1	CCTCAACACATAGCAAATCG			

2.3 DNA preparation

The DNA of the individual worms was extracted using the DNeasy® Blood & Tissue Kit 250 (QIAGEN, Hilden, Germany), following the instructions given by the manufacturer. In brief, 1 cm of worm was placed in a 1.5 ml reaction tube and 180 µL ATL buffer was added. After adding 20 µL Proteinase K, the tube was mixed thoroughly by vortexing and incubated at 56°C until the sample had been lysed completely. To increase lysing speed, the sample was vortexed during incubation regularly. After the sample had been completely dissolved, 200 µL ATL buffer were added before incubating it in a thermo-mixer at 70°C for 10 minutes. The tube was then spun down to remove drops from the lid and 200 µL 100% ethanol was added. The sample was then again vortexed before its content was applied onto a spin column and centrifuged at 8,000 rpm for 1 minute. The column was placed in a clean tube and washed with 500 µL AW1 buffer before it was centrifuged at 8,000 rpm for 1 minute. The column was then again placed into a clean tube and 500 µL AW2 buffer was added onto the column before centrifuging it at 14,000 rpm for 3 minutes. The flow-through was discarded and the tube was again centrifuged for 1 minute to ensure that no washing buffer remained inside. Subsequently, the column was placed in a clean 1.5 mL reaction tube and 50 µL AE buffer were added onto the column, before incubating it for 1 minute at room temperature. Finally, the sample was centrifuged at 8,000 rpm for 1 minute, giving us our DNA sample. Extracted samples were stored at -20°C.

2.4 PCRs

All samples were prepared in a standardized manner for DNA amplification. A reaction volume of 50 µL containing the DNA and the respective primers was set up for each sample. The master mix consisted of magnesium-free reaction buffer (Solis BIODYNE), $MgCl_2$ (25 mM), deoxynucleoside triphosphate (dNTP) mix (20 mM, Solis BIODYNE), double-distilled water (ddH_2O), forward primer (10 pmol/µl), reverse primer (10 pmol/µl) and Hot FIREPol DNA polymerase (5 U/µl, Solis BioDyne, Tartu, Estonia). The exact composition of the reaction volume is shown in Table 3. Amplification was performed with varying amounts of DNA to ensure high signal strength of the PCR results. All pipetting work was performed on a sterile workbench to prevent sample contamination. A negative control consisting of 32.25 µL master mix and 17.75 µL ddH_2O was included in each PCR run.

Table 3. PCR master mix and reaction volume composition.

Components	Volume
Reaction buffer B (Mg^{2+} free)	5 µL
$MgCl_2$	5 µL
Primer I (forward)	5 µL
Primer II (reverse)	5 µL
ddH_2O	11 µL
dNTPs	1 µL
DNA polymerase	0.25 µL
Master mix	**32.25 µL**
DNA	1 – 3 µL
Adding ddH_2O to final volume	**50 µL**

Table 4. PCR conditions for all target loci.

Target	Cycles	PCR step	Temperature	Time	Primer
cox1		Initial denaturation	95°C	15 min	As-Co1F As-Co1R
	30	Denaturation	94°C	30 sec	
		Annealing	55°C	30 sec	
		Extension	72°C	30 sec	
		Final extension	72°C	7 min	
nad1		Initial denaturation	95°C	15 min	MH5F As-NDR
	30	Denaturation	94°C	30 sec	
		Annealing	55°C	30 sec	
		Extension	72°C	30 sec	
		Final extension	72°C	7 min	
		Initial denaturation	95°C	15 min	MH5F As-NDR
	35	Denaturation	94°C	1 min	
		Annealing	48°C	1 min	
		Extension	72°C	1 min	
		Final extension	72°C	7 min	
ITS1		Initial denaturation	95°C	15 min	F2662 R3214 As NF-ITS1 As NR-ITS1
	45	Denaturation	92°C	1 min	
		Annealing	52°C	1 min	
		Extension	72°C	1 min	
		Final extension	72°C	7 min	
All		Resting	10°C	x	All

All PCRs were run in a Mastercycler ep Gradient S (Eppendorf, Hamburg, Germany). The PCR conditions corresponded to the protocols of Peng et al. (2005) for *cox1* and *nad1*, and Ishiwata et al. (2004) for ITS1. Since samples could not be amplified successfully for *nad1* using the PCR protocol of Peng et al. (2005), the program was adjusted during the study. It was not necessary to adjust the ITS1 PCR program for the new ITS1 primers. The exact PCR conditions and corresponding primers are shown in Table 4.

2.5 Gel electrophoresis

PCR amplification was analyzed via gel electrophoresis on a 2% agarose gel. For this, 2 g of agarose (Sigma-Aldrich, St. Louis, USA) was dissolved in 100 ml of TAE buffer (0.04 M Tris acetate, 0.0001 M EDTA) and heated on a heating plate using a magnetic stirrer rod until the agarose had dissolved. After the mixture had cooled down to 60°C, 5 µL GelRed™ (Biotium, Fremont, USA) were added and stirred in. The liquid was then poured into a gel tray equipped with a well comb. After approximately 30 minutes, the gel was completely polymerized and was then loaded with the samples.

For loading onto the gel, 25 µL of PCR product was mixed with 5 µL of gel loading buffer (Sigma Aldrich, St. Louis, USA) before being loaded onto the agarose gel. 30 µL of a 50 bp DNA step ladder (Sigma Aldrich, St. Louis, USA) was loaded onto each gel to evaluate the size of the amplified DNA fragments. In addition, 30 µL of negative control, consisting of 25 µL negative control mixture and 5 µL gel loading buffer, was added to each run, enabling detection of contamination and amplification errors.
The prepared gel was then placed into a BIO-RAD Wide Mini-Sub Cell GT electrophoresis chamber (Bio-Rad Laboratories, Inc., California, USA) and run at 134 volts and 300 mA for about 30 minutes, until the loading buffer had reached the end of the gel.

2.6 Gel band analysis and purification

The bands produced by electrophoresis were visualized using UV light in a transilluminator (Gel Doc XR+, Bio-Rad Laboratories, Inc., California, USA) and checked for appropriate sizes. The respective DNA bands were cut out of the gel using a sterile scalpel and placed into separate 1.5 mL reaction tubes.
Subsequently, 300 µL capture buffer was added to the test tubes and the bands were purified

using the GFX™ PCR DNA and Gel Band Purification Kit (Cytiva Europe GmbH, Vienna, Austria) according to the manufacturer's protocol. Depending on the strength of the bands, the DNA was eluted from the columns with 20–50 µL elution buffer, incubated for 1 minute, and then centrifuged at 14,000 rpm for 30 seconds. The samples were stored at -20°C.

2.7 DNA sequencing

For DNA sequencing, the PCR was standardized for all samples. In brief, 10 µL sequencing reactions were prepared from each sample, consisting of one primer (forward or reverse), AB mix (BigDye™ Terminator v1.1 Cycle Sequencing RR-100, Applied Biosystems, Thermo Fisher Scientific, Waltham, USA), sequencing buffer (BigDye® Terminator v1.1, v.3.1, Applied Biosystems, Thermo Fisher Scientific, Waltham, USA), purified PCR amplicons, and sterile H_2O. The exact composition of the reaction volume is shown in Table 5. The sequencing PCR conditions were identical for all primers and are shown in Table 6. Sequences were obtained from both strands in at least two independent getups.

Table 5. Sequencing PCR reaction composition.

Components	Volume
Primer (forward or reverse)	2 µL
AB-mix	2 µL
Sequencing buffer	1 µL
PCR-product	1 - 3 µL
Adding H_2O to end volume	**10 µL**

Table 6. Sequencing PCR conditions.

Target	Cycles	PCR step	Temperature	Time	Primer
All		Incubation	96°C	30 sec	As-Co1F As-Co1R MH5F As-NDR F2662 R3214 As NF-ITS1 As NR-ITS1
	30	Denaturation	96°C	10 sec	
		Annealing	50°C	5 sec	
		Extension	60°C	4 min	
		Hold	4°C	x	

The sequencing products were transferred to micro test tubes containing 1 µL sodium acetate (CH_3COONa) and mixed with 40 µL 100% alcohol before being incubated on ice for 17 minutes. After incubation, the samples were centrifuged for 30 minutes at 4°C and 12,000 x g (Heraeus FrescoTM 17 Centrifuge, Thermo Fisher Scientific, Waltham, USA). The supernatant was then removed without disturbing the pellet formed during centrifugation. After adding 90 µl of 70% alcohol, the centrifugation process was repeated for 10 minutes under the previous conditions. After removing the supernatant, the samples were left open for 3 minutes to allow residual alcohol to evaporate. After adding 20 µl of HiDi™ formamide (Applied Biosystems, Thermo Fisher Scientific, Waltham, USA), the test tubes were left open for 5 minutes before being placed into a thermomixer (Thermomixer comfort 1.5 ml, Eppendorf AG, Hamburg, Germany), being incubated at 95°C for 4 minutes. After subsequent incubation on ice for 10 minutes, the samples were transferred to sequencing strips and were then placed into a SeqStudio Genetic Analyzer (Applied Biosystems, Thermo Fisher Scientific, Waltham, USA) for sequencing.

2.8 Sequence alignments and data analyses

Resulting sequences were checked for lengths and quality before being blasted and compared with sequences from the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Individual misidentified or missing bases were corrected based on the consensus using Chromas Lite software, version 1.62 (Technelysium Pty Ltd, 2025). Multiple sequences of one target from both strands were then combined into consensus sequences using GeneDoc (National Resource for Biomedical Supercomputing, Pittsburgh, USA). Consensus sequences were compared with 50 reference sequences of *Ascaris* spp. from GenBank (Tab. 7) and aligned using the ClustalX program (Conway Institute, Dublin, Ireland). To ensure comparability, reference sequences were selected from various studies with varying sampling locations and chosen such that all sequences were of equal or greater length than the consensus sequences. Sequences of questionable quality were not included.

Table 7. Reference sequences used for *cox1* comparison, with corresponding GenBank accession numbers, species identification, host, country of origin and author.

cox1				
GenBank No.	**Species**	**Host**	**Country**	**Author**
AB591802.1	A. suum	pig	Japan	Arizono et al., 2010
AB591803.1	A. suum	pig	Japan	Arizono et al., 2010
AB591804.1	A. suum	pig	Japan	Arizono et al., 2010
AJ968335.1	A. suum	pig	China	Peng et al., 2005
AJ968336.1	A. suum	pig	China	Peng et al., 2005
AJ968337.1	A. suum	pig	China	Peng et al., 2005
AJ968340.1	A. suum	pig	China	Peng et al., 2005
AJ968341.1	A. suum	pig	China	Peng et al., 2005
AJ968343.1	A. suum	pig	China	Peng et al., 2005
HM602025.1	A. suum	pig	Brazil	Leles et al., 2010
KF719122.1	A. suum	pig	Uganda	Betson et al., 2014
KF719125.1	A. suum	pig	Uganda	Betson et al., 2014
KF719127.1	A. suum	pig	Denmark	Betson et al., 2014
KF719128.1	A. suum	pig	Guatemala	Betson et al., 2014
KF719131.1	A. suum	pig	Tanzania	Betson et al., 2014
KF719135.1	A. suum	pig	Tanzania	Betson et al., 2014
KF719141.1	A. suum	pig	United Kingdom	Betson et al., 2014
KF719142.1	A. suum	pig	United Kingdom	Betson et al., 2014
MF358905.1	A. suum	pig	Laos	Sadaow et al., 2018
MF358911.1	A. suum	pig	Thailand	Sadaow et al., 2018
MF358931.1	A. suum	pig	Thailand	Sadaow et al., 2018
MF358933.1	A. suum	pig	Thailand	Sadaow et al., 2018
MK143378.1	A. suum	pig	Brazil	Monteiro et al., 2019
MK143379.1	A. suum	pig	Brazil	Monteiro et al., 2019
MK143381.1	A. suum	pig	Brazil	Monteiro et al., 2019
AB591797.1	A. lumbricoides	human	Japan	Arizono et al., 2010
AJ968324.1	A. lumbricoides	human	China	Peng et al., 2005
AJ968332.1	A. lumbricoides	human	China	Peng et al., 2005
AJ968333.1	A. lumbricoides	human	China	Peng et al., 2005
EU582486.1	A. lumbricoides	human	Zanzibar	Betson et al., 2011
EU582493.1	A. lumbricoides	human	Zanzibar	Betson et al., 2011
EU582499.1	A. lumbricoides	human	Zanzibar	Betson et al., 2011
GU326948.1	A. lumbricoides	human	Brazil	Leles et al., 2009
GU326953.1	A. lumbricoides	human	Brazil	Leles et al., 2009
GU326955.1	A. lumbricoides	human	Brazil	Leles et al., 2009

KF536871.1	A. lumbricoides	human	Uganda	Betson et al., 2012
KF719094.1	A. lumbricoides	human	Bangladesh	Betson et al., 2014
KF719118.1	A. lumbricoides	human	Uganda	Betson et al., 2014
KM365023.1	A. lumbricoides	human	Denmark	Søe et al., 2015
LN600400.1	A. lumbricoides	pig	Czech Republic	Civáňová Křížová et al., 2023
MF358908.1	A. lumbricoides	human	Laos	Sadaow et al., 2018
MF358914.1	A. lumbricoides	human	Thailand	Sadaow et al., 2018
MF358935.1	A. lumbricoides	human	Thailand	Sadaow et al., 2018
MH674442.1	A. lumbricoides	human	Brazil	Monteiro et al., 2018
MH800229.1	A. lumbricoides	human	Brazil	Monteiro et al., 2018
MH800277.1	A. lumbricoides	human	Brazil	Monteiro et al., 2018
MH800281.1	A. lumbricoides	human	Brazil	Monteiro et al., 2018
MK792796.1	A. lumbricoides	human	Malaysia	Mohd-Shaharuddin et al., 2019
MK792798.1	A. lumbricoides	human	Malaysia	Mohd-Shaharuddin et al., 2019
MK792812.1	A. lumbricoides	human	Malaysia	Mohd-Shaharuddin et al., 2019

Table 8. Reference sequences used for *nad1* comparison, with corresponding GenBank accession numbers, species identification, host, country of origin and author

nad1				
GenBank No.	**Species**	**Host**	**Country**	**Author**
AJ968355.1	A. suum	pig	China	Peng et al., 2005
AJ968356.1	A. suum	pig	China	Peng et al., 2005
AJ968357.1	A. suum	pig	China	Peng et al., 2005
AJ968358.1	A. suum	pig	China	Peng et al., 2005
AJ968359.1	A. suum	pig	China	Peng et al., 2005
AJ968360.1	A. suum	pig	China	Peng et al., 2005
AJ968363.1	A. suum	pig	China	Peng et al., 2005
AJ968364.1	A. suum	pig	China	Peng et al., 2005
AJ968365.1	A. suum	pig	China	Peng et al., 2005
AJ968366.1	A. suum	pig	China	Peng et al., 2005
AJ968368.1	A. suum	pig	China	Peng et al., 2005
AJ968369.1	A. suum	pig	China	Peng et al., 2005
GU326964.1	A. suum	pig	Brazil	Leles et al., 2009
HM602026.1	A. suum	pig	Brazil	Leles et al., 2010
HM602027.1	A. suum	pig	Brazil	Leles et al., 2010
HM602028.1	A. suum	pig	Brazil	Leles et al., 2010
HQ704901.1	A. suum	pig	China	Liu et al., 2012

KC998843.1	A. suum	pig	China	Chang et al., 2015
KY045800.1	A. suum	pig	Tanzania	Nejsum et al., 2017
KY045804.1	A. suum	pig	United Kingdom	Nejsum et al., 2017
KY045805.1	A. suum	pig	Uganda	Nejsum et al., 2017
MK160501.1	A. suum	pig	Brazil	Monteiro et al., 2019
MK160502.1	A. suum	pig	Brazil	Monteiro et al., 2019
MK160503.1	A. suum	pig	Brazil	Monteiro et al., 2019
NC_001327.1	A. suum	pig	USA	Wolstenholme et al., 1994
AJ968346.1	A. lumbricoides	human	China	Peng et al., 2005
AJ968347.1	A. lumbricoides	human	China	Peng et al., 2005
AJ968352.1	A. lumbricoides	human	China	Peng et al., 2005
AJ968353.1	A. lumbricoides	human	China	Peng et al., 2005
GU326956.1	A. lumbricoides	human	Brazil	Leles et al., 2009
GU326957.1	A. lumbricoides	human	Brazil	Leles et al., 2009
GU326960.1	A. lumbricoides	human	Brazil	Leles et al., 2009
GU326962.1	A. lumbricoides	human	Brazil	Leles et al., 2009
KX022399.1	A. lumbricoides	human	Brazil	Alves et al., 2016
KX022400.1	A. lumbricoides	human	Brazil	Alves et al., 2016
MK160504.1	A. lumbricoides	human	Brazil	Monteiro et al., 2019
MK160506.1	A. lumbricoides	human	Brazil	Monteiro et al., 2019
MK160510.1	A. lumbricoides	human	Brazil	Monteiro et al., 2019
MK160512.1	A. lumbricoides	human	Brazil	Monteiro et al., 2019
MK160518.1	A. lumbricoides	human	Brazil	Monteiro et al., 2019
MK160526.1	A. lumbricoides	human	Brazil	Monteiro et al., 2019
MK160529.1	A. lumbricoides	human	Brazil	Monteiro et al., 2019
MK160532.1	A. lumbricoides	human	Brazil	Monteiro et al., 2019
MK160536.1	A. lumbricoides	human	Brazil	Monteiro et al., 2019
MK160540.1	A. lumbricoides	human	Brazil	Monteiro et al., 2019
MK160547.1	A. lumbricoides	human	Brazil	Monteiro et al., 2019
MK160549.1	A. lumbricoides	human	Brazil	Monteiro et al., 2019
MK160558.1	A. lumbricoides	human	Brazil	Monteiro et al., 2019
MK160562.1	A. lumbricoides	human	Brazil	Monteiro et al., 2019
MK160568.1	A. lumbricoides	human	Brazil	Monteiro et al., 2019

Table 9. Reference sequences used for ITS1 comparison, with corresponding GenBank accession numbers, species identification, host, country of origin and author

ITS1				
GenBank No.	**Species**	**Host**	**Country**	**Author**
MF358943.1	A. suum	pig	Laos	Sadaow et al., 2018
KY964445.1	A. suum	pig	China	Li, 2017
KY964446.1	A. suum	pig	China	Li, 2017
AB571302.1	A. suum	pig	Japan	Arizono et al., 2010
OQ825949.1	A. suum	pig	Myanmar	Bawm et al., 2024
KY964447.1	A. suum	pig	China	Li, 2017
KY964444.1	A. suum	pig	China	Li, 2017
MF358944.1	A. suum	pig	Thailand	Sadaow et al., 2018
MF358937.1	A. suum	pig	Thailand	Sadaow et al., 2018
MF358950.1	A. suum	pig	Laos	Sadaow et al., 2018
MF358947.1	A. suum	pig	Thailand	Sadaow et al., 2018
HQ721824.1	A. suum	pig	China	Wu et al., 2010
HQ721825.1	A. suum	pig	China	Wu et al., 2010
HQ721826.1	A. suum	pig	China	Wu et al., 2010
AB110023.1	A. suum	pig	Japan	Ishiwata et al., 2004
HQ721827.1	A. suum	pig	China	Wu et al., 2010
AJ000896.1	A. suum	pig	unknown	Zhu et al., 1999
MF358940.1	A. lumbricoides	human	Myanmar	Sadaow et al., 2018
MF358952.1	A. lumbricoides	human	Myanmar	Sadaow et al., 2018
MF358948.1	A. lumbricoides	human	Myanmar	Sadaow et al., 2018
ON678066.1	A. lumbricoides	human	Iraq	Hassan, 2022
ON678068.1	A. lumbricoides	human	Iraq	Hassan, 2022
ON678067.1	A. lumbricoides	human	Iraq	Hassan, 2022
MF358938.1	A. lumbricoides	human	Myanmar	Sadaow et al., 2018
ON678065.1	A. lumbricoides	human	Iraq	Hassan, 2022
LC422641.1	A. lumbricoides	human	North Korea	Sato et al., 2019
MK849917.1	A. lumbricoides	human	Iraq	Al-Mozan, 2019
MK849920.1	A. lumbricoides	human	Iraq	Al-Mozan, 2019
MK849918.1	A. lumbricoides	human	Iraq	Al-Mozan, 2019
MK849916.1	A. lumbricoides	human	Iraq	Al-Mozan, 2019
MK849919.1	A. lumbricoides	human	Iraq	Al-Mozan, 2019
AB576588.1	A. lumbricoides	human	Japan	Arizono et al., 2010
AB571298.1	A. lumbricoides	human	Japan	Arizono et al., 2010
AB571297.1	A. lumbricoides	human	Japan	Arizono et al., 2010
HQ721820.1	A. lumbricoides	human	China	Wu et al., 2010

AB110021.1	A. lumbricoides	human	Bangladesh	Ishiwata et al., 2004
AB110019.1	A. lumbricoides	human	Japan	Ishiwata et al., 2004
AB571295.1	A. lumbricoides	human	Japan	Arizono et al., 2010
EF153619.1	A. lumbricoides	human	Brazil	Leles et al., 2009
HQ721821.1	A. lumbricoides	human	China	Liu et al., 2012
LC422642.1	A. lumbricoides	human	North Korea	Sato et al., 2019
LC422643.1	A. lumbricoides	human	North Korea	Sato et al., 2019
OQ539679.1	A. lumbricoides	human	Indonesia	Civáňová Křížová et al., 2023
MF358959.1	A. lumbricoides	human	Laos	Sadaow et al., 2018
MF358960.1	A. lumbricoides	human	Thailand	Sadaow et al., 2018
MF358963.1	A. lumbricoides	human	Thailand	Sadaow et al., 2018
MF358941.1	A. lumbricoides	human	Thailand	Sadaow et al., 2018
MF358945.1	A. lumbricoides	human	Laos	Sadaow et al., 2018
MF358949.1	A. lumbricoides	human	Thailand	Sadaow et al., 2018
MF358951.1	A. lumbricoides	human	Laos	Sadaow et al., 2018

Finally, sequences were trimmed, to remove PCR primer regions and ambiguous sites, to final lengths of 383 bp for *cox1*-sequences, 357 bp for *nad1*-sequences and 450 bp for ITS1-sequences respectively. Multiple sequence alignment and the calculation of dissimilarity matrices were performed using MEGA11: Molecular Evolutionary Genetics Analysis, version 11 (Tamura et al., 2021) for *cox1* and *nad1*, using the ClustalW alignment algorithm (Thompson et al., 1994), and using DnaSP, version 6.12.03 (Rozas et al., 2017) for ITS1. Phylogenetic relationships between the *cox1* and *nad1* haplotypes were determined using MEGA11, while ITS1 haplotypes were analyzed using DnaSP. Haplotype networks were generated using the Median-Joining method and visualized in PopART: Population Analysis with Reticulate Trees, version 1.7 (https://popart.maths.otago.ac.nz/) for *cox1* and *nad1* networks, and Fluxus Network, version 10.2.0.0 (fluxus-engineering.com) for the ITS1 network (Bandelt et al., 1999). Fluxus Network was selected due to gaps in the ITS1 comparison sequences, treating these as separate mutations, rather than ignoring them. For *cox1* and *nad1*, this was not necessary.

2.9 Use of Artificial Intelligence Tools

Artificial intelligence-based tools were used during the preparation of this manuscript. ChatGPT, version GPT-5.3 (OpenAI, 2026), was used to assist with improving the text structure, refining linguistic phrasing and brainstorming. DeepL Translator (DeepL SE, 2026) was used to assist with translation and to improve wording by suggesting alternative expressions. The literature research software ResearchRabbit (ResearchRabbit, 2025) was used to assist in the search for relevant literature. The results from these tools were critically reviewed, checked for accuracy and edited. Suggested references were verified before being used.

3. Results

3.1 PCR results

The *cox1* and ITS1 were successfully amplified from all nine Austrian *Ascaris* isolates. All *cox1* gene sequences were amplified according to the protocol described by Peng et al. (2005); for ITS1 (Ishiwata et al., 2004) additional primers had to be used, as shown in Table 2. For *nad1*, amplicons could only be successfully generated for seven samples. Since *nad1* amplification using the PCR conditions specified by Peng et al. (2005) was entirely unsuccessful, cycler conditions were modified as described in Table 4. An overview of all PCR results, including sample age and known travel history, is provided in Table 8.

Table 10. Overview of PCR results. Successful (**+**) and unsuccessful (**-**) amplifications of marker loci are shown, together with details on sample age and travel history.

Sample name	Year of isolation	cox1 amplification	nad1 amplification	ITS1 amplification	Travel history
10A1	2010	+	+	+	no data
10A2	2010	+	-	+	no data
16A1	2016	+	+	+	Indonesia, Singapore, Thailand
18A1	2018	+	+	+	no data
18A2	2018	+	-	+	no travel
19A1	2019	+	+	+	Indonesia
23A1	2023	+	+	+	no data
24A1	2024	+	+	+	Bulgaria
24A2	2024	+	+	+	no data

Cox1 amplification (Fig. 8) was robust with a low error rate and clear and strong bands. The amplicon length was approximately 400 bp prior to trimming.

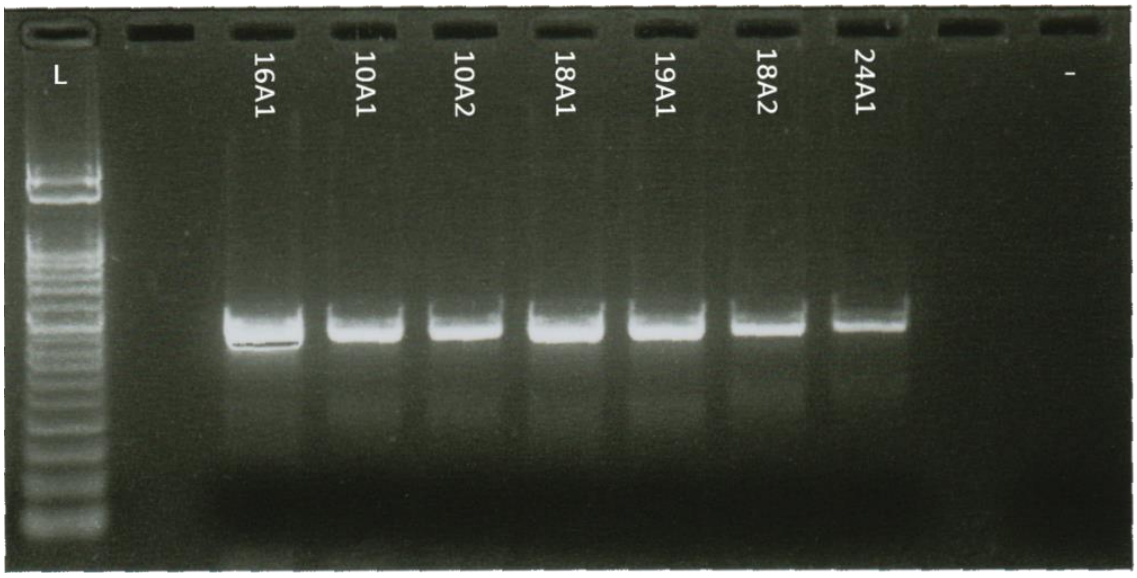

Figure 8. Example of *cox1* PCR results. Slots are marked with sample names. Unmarked slots were left empty. L = ladder marker; - = negative control.

Amplification of the *nad1* gene using the original cycling conditions yielded no results. Thus, the cycling conditions were changed (Tab. 4). This adjustment enabled successful amplification of samples 10A1, 16A1, 18A1, 19A1, 23A1, 24A1 and 24A2 (Fig. 9). However, samples 10A2 and 18A2 consistently failed to amplify despite multiple attempts. Amplicon length was approximately 380 bp before trimming.

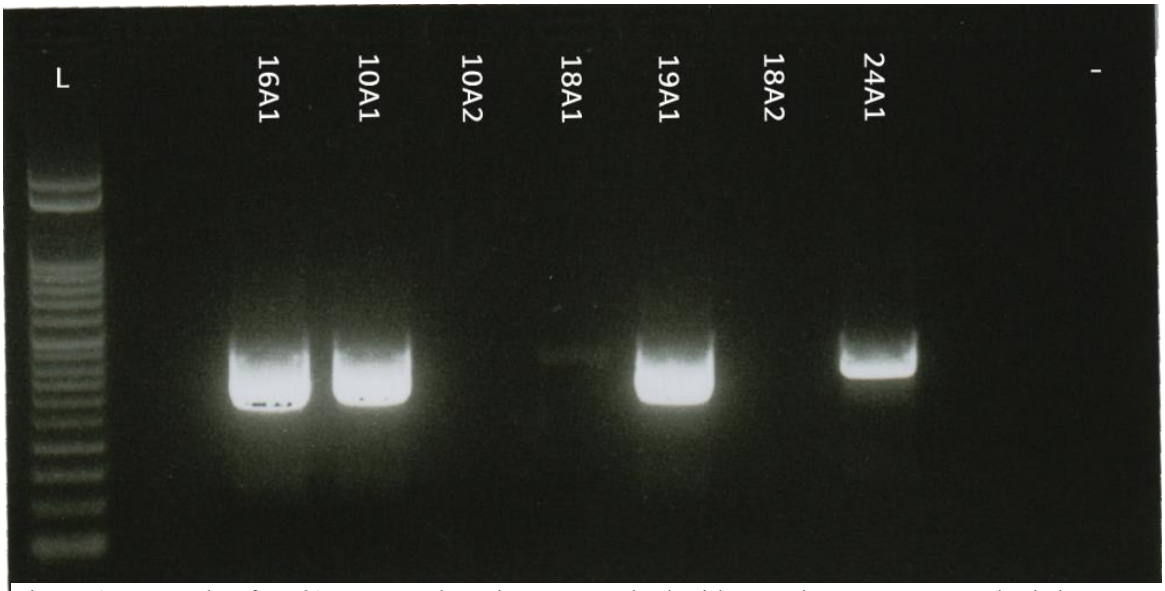

Figure 9. Example of *nad1* PCR results. Slots are marked with sample names. Unmarked slots were left empty. Samples 10A2 and 18A2 did not result in any visible bands. Sample 18A1 gave a very faint band. L = ladder marker; - = negative control.

The ITS1 PCR using the protocol of Ishiwata et al. (2004) resulted in clear and uniform bands (Fig. 10). The amplicon length was approximately 460 bp before trimming. However, evaluation of the obtained DNA sequences revealed that some ITS1 forward sequences contained significant sequencing errors in the A and T rich regions close to the 5′-end. Because of the otherwise good quality of the sequences obtained, it was decided to perform partial amplification of the 5' and 3' ends with the newly designed As NF-ITS1 and As NR-ITS1 primers, paired with the previous primers as forward or reverse counterparts (Fig. 11) instead of complete resequencing. The resulting sequences enabled the assembly of consensus sequences for further analysis. Amplification with As NF-ITS1 resulted in the amplification of unspecified DNA fragments in the negative control (Fig. 11). Sequencing of these revealed a fungal origin (*Neoascochyta* spp.).

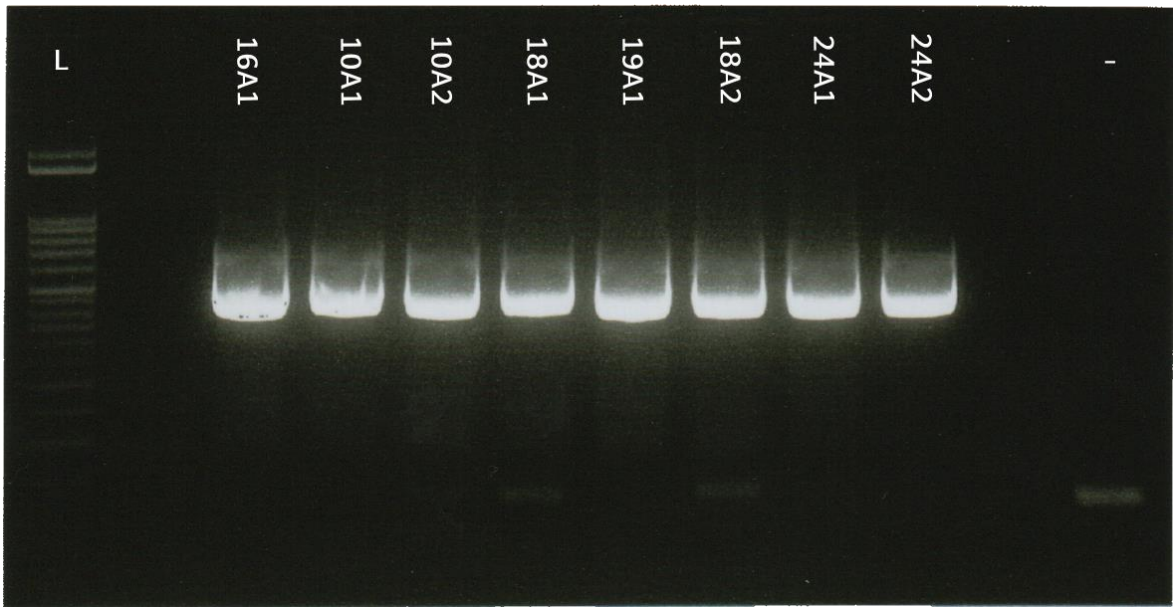

Figure 10. Example of ITS1 PCR results with primers F2662 and R3214. Slots are marked with sample names. Unmarked slots were left empty. L = ladder marker; - = negative control.

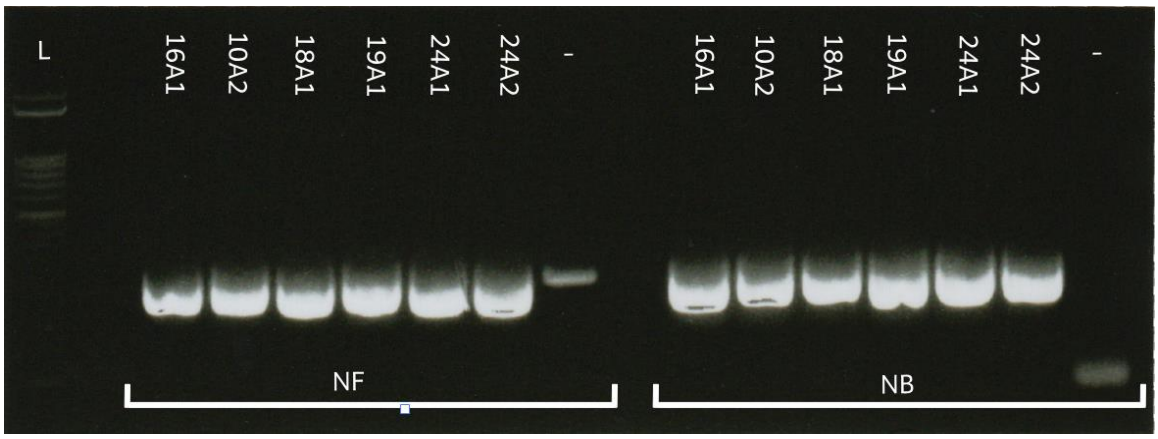

Figure 11. Example of ITS1 PCR results with primers F2662 and As NR-ITS1 (**NF**), and R3214 and As NF-ITS1 (**NB**). Slots are marked with sample names. Unmarked slots were left empty. L = ladder marker; - = negative control.

3.2 Alignments

The target loci *cox1* and ITS1 were successfully sequenced for all *Ascaris* samples, while sequencing of *nad1* was successful in seven samples. The alignments for the different genetic loci of the sequences of all samples investigated in this study are shown in figures 12-14.

Alignment of the *cox1* sequences (383 bp) identified 24 variable (6.3%) and 359 conserved (93.7%) positions among the Austrian isolates. Pairwise comparisons revealed sequence dissimilarity ranging from 0 to 21 base pairs (0-5.5%), with isolates 16A1, 19A1 and 23A1 showing 100% (383bp/383bp) sequence identity.

For *nad1* (357 bp), 21 variable (5.9%) and 336 conserved (94.1%) positions were detected. Pairwise comparisons showed sequence dissimilarity ranging from 1 to 19 base pairs (0.3-5.3%) and no isolates showed 100% sequence identity with another.

In the ITS1 alignment (450 bp), with alignment gaps (-) treated as nucleotide positions, 12 variable (2.7%) and 438 conserved (97.3%) positions were identified. Pairwise comparisons revealed 0 to 10 base pair differences (0-2.2%). Isolates 16A1, 18A2 and 19A1 showed 100% (450bp/450bp) sequences identity, as well as isolates 24A1 and 24A2.

When 50 reference sequences were included in the analysis, the observed sequence divergence increased. The *cox1* alignment showed 0-23 nucleotide differences (0-6%), the *nad1* alignment 0-21 nucleotide differences (0-6%) and the ITS1 alignment 0-9 nucleotide differences (0-2%).

Alignments of the *cox1* and *nad1* sequences and the comparison with reference sequences did not allow discrimination between different *Ascaris* species. In contrast, ITS1 sequence alignment allowed for differentiation between *A. lumbricoides* and *A. suum*, following the criteria described by Sadaow et al. (2018). Differing nucleotides at positions 5, 6, 130, and 244 (Fig. 14) were used as markers for species identification. Based on these, samples 10A1 and 23A1 were classified as *A. lumbricoides*; all remaining samples as *A. suum*. Both *A. lumbricoides* samples came from patients with unknown travel history.

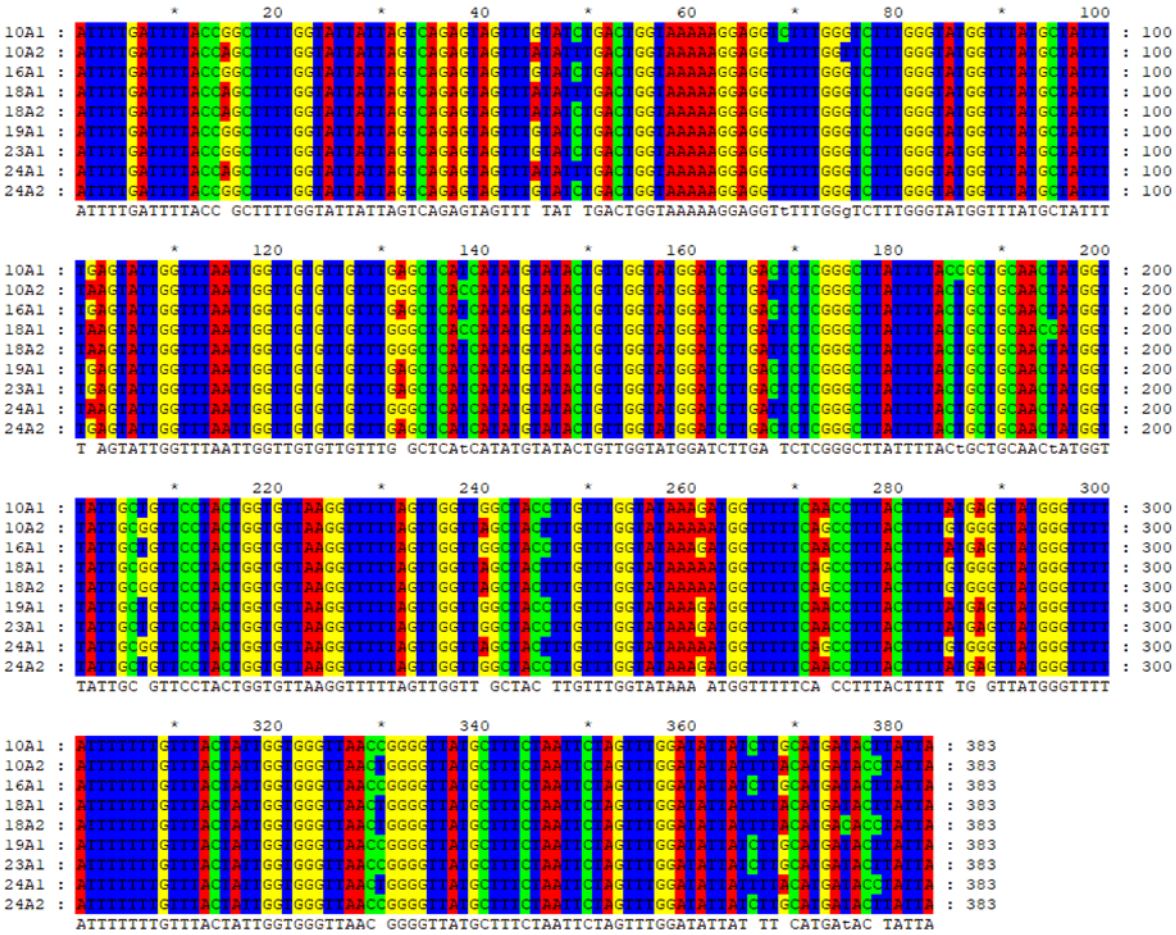

Figure 12. Alignment of all *cox1* sequences obtained in this study. Sequences were trimmed to 383 bp length.

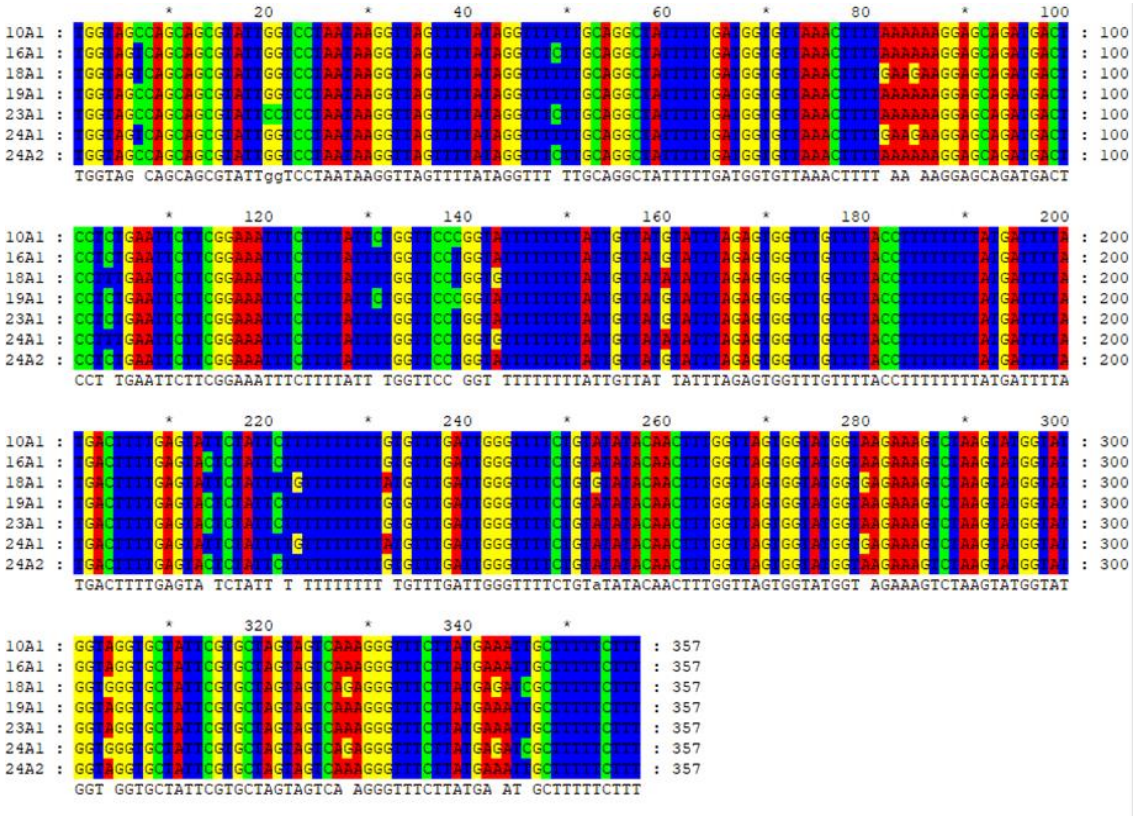

Figure 13. Alignment of all *nad1* sequences obtained in this study. Sequences were trimmed to 357 bp length.

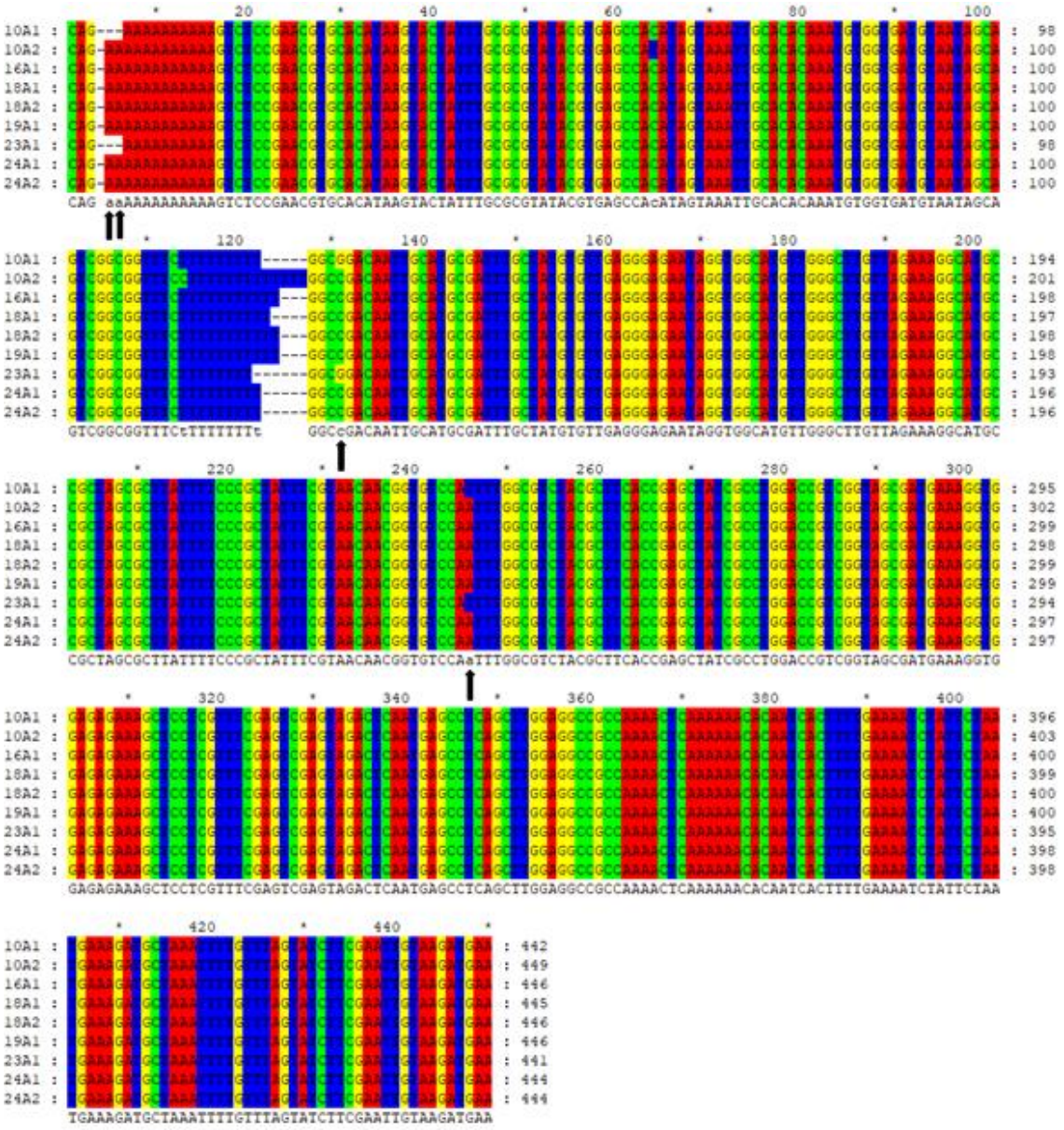

Figure 14. Alignment of all ITS1 sequences obtained in this study. Sequences were trimmed to 450 bp length. Positions differing between *A. lumbricoides* and *A. suum* (5, 6, 130, 244) as described by Sadaow et al. (2018) are marked by arrows.

3.3 Haplotype analyses and networks

Altogether, six *cox1* haplotypes, seven *nad1* haplotypes, and six ITS1 haplotypes were identified among our *Ascaris* samples.

The ***cox1* haplotype** network was constructed including 50 reference sequences. In total, 40 *cox1* haplotypes (cH) were detected across all sequences and were grouped into three clusters: cluster A, B and C. The nine Austrian isolates were assigned to six haplotypes: one isolate to cH5 (10A1), 4 isolates to cH21 (16A1, 19A1, 23A1, 24A2), one isolate to cH37 (18A2), one isolate to cH38 (10A2), one isolate to cH39 (24A1) and one isolate to cH40 (18A1). Five of the isolates were assigned to haplotypes within cluster A (10A1, 16A1, 19A1, 23A1, 24A2), while four were located in cluster C (10A2, 18A1, 18A2, 24A1). None of the isolates grouped within cluster B. Of the three samples from patients with reported travel history, two samples assigned to haplotype cH21 (16A1, 19A1), located in cluster A and were from patients with travel history to Indonesia. The single sample identified as haplotype cH39 (24A1), located in cluster C, originated from a patient with travel history to Bulgaria. The sample without travel history (18A2) had haplotype cH37, belonging to cluster C. Despite aggregation of the sequences into three clusters, the *cox1* network does not separate *A. lumbricoides* and *A. suum*. Clusters contain haplotypes from both, *A. lumbricoides* and *A. suum*, as well as shared haplotypes that contain both species. Austrian isolates were identified as part of shared haplotype groups (cH5, cH21) within cluster A, and as separate haplotypes within cluster C (cH37-cH40). Consequently, the *cox1* network does not allow clear species differentiation (Fig. 15).

A similar pattern was observed in the ***nad1* haplotype** network. A total of 42 *nad1* haplotypes (nH) were detected across all sequences, including the 50 reference sequences. These haplotypes grouped into three clusters: A, B and C. Each of the seven successfully sequenced isolates was assigned to a distinct haplotype, with isolates 16A1, 10A1, 24A2, 24A1, 18A1, 19A1 and 23A1 corresponding to haplotypes nH37, nH38, nH39, nH40, nH41, nH42 and nH43, respectively. Five of the isolates were assigned to haplotypes within cluster A (10A1, 16A1, 19A1, 23A1, 24A2), while two were located in cluster C (18A1, 24A1). None of the isolates grouped within cluster B. Clusters A and B share haplotypes of *A. lumbricoides* and *A. suum*, as in the *cox1* network, with the exception of cluster C, only containing two haplotypes of Austrian isolates and one of *A. suum*. The three samples from patients with reported travel history were assigned to distinct haplotypes (nH37, nH40, nH42). Samples 16A1 and 19A1 clustered within cluster A, despite exhibiting genetic

distance from one another, whereas isolate 24A1 and its associated haplotype nH40 were assigned to cluster C. Although cluster C did not contain haplotypes from multiple species, the presence of only a single reference haplotype is insufficient for reliable species differentiation. Consequently, the *nad1* network likewise does not allow clear species separation (Fig. 16).

In contrast to the mitochondrial markers, the **ITS1 haplotype** network showed a clear separation into two clusters, A and B. In total, 21 ITS1 haplotypes (iH) were identified across all sequences, including 50 reference sequences. Cluster A was dominated by *A. lumbricoides* reference haplotypes, whereas cluster B comprised of *A. suum* reference haplotypes, both separated along the branch between haplotypes iH14 and iH4. The nine Austrian isolates were assigned to six haplotypes: one isolate to iH1 (18A1), one isolate to iH13 (10A1), one isolate to iH18 (23A1), three isolates to iH19 (16A1, 18A2, 19A1), two isolates to iH20 (24A1, 24A2) and one isolate to iH21 (10A2). Isolates 10A1 and 23A1 were assigned to cluster A associated with *A. lumbricoides*, whereas the remaining isolates grouped within cluster B associated with *A. suum*. Haplotype iH21 appeared as an outlier. The two isolates from patients with reported travel history to Indonesia (16A1, 19A1) shared haplotype iH19, together with the isolate with no travel history (18A2). These samples, together with the isolate with travel history to Bulgaria (24A2) and its associated haplotype iH20, clustered within cluster B. In accordance with the species separation observed in the ITS1 sequence alignment, samples 10A1 and 23A1 were assigned to cluster A associated with *A. lumbricoides*, with isolate 10A1 being part of a larger shared haplotype group (iH13) and sample 23A1 representing a closely related haplotype within the same cluster. The remaining samples were assigned to cluster B associated with *A. suum*, with sample 18A1 sharing a haplotype group with reference sequences. The other Austrian isolates formed closely related haplotype groups without reference sequences (iH19, iH20) or the outlier haplotype iH21 (Fig. 17).

Comparison of the three haplotype networks revealed that isolates did not consistently group together across markers. The *A. suum* associated samples 10A1 and 23A1 grouped closely in the *cox1* and ITS1 network, yet showed no close genetic relationship to each other in the mitochondrial *nad1* network. A similar pattern was observed when examining isolates with known travel history. Although sample 23A1 shared a *cox1* haplotype with isolates from patients who had traveled to Indonesia, the *nad1* network showed that these isolates were more genetically distinct. The ITS1 network further indicated that 23A1 was

differentiated from the travel-associated samples at the species level. In contrast, isolates from patients with reported travel history showed some genetic separation in the mitochondrial networks, spreading across different clusters, but clustered closely together in the *A. suum* associated ITS1 Cluster B, sharing haplotypes with one another. Interestingly, isolate 18A2 was also found in haplotype iH19, despite isolate 18A2 having no travel history.

Table 9 provides an overview of the haplotypes identified among the Austrian isolates, indicating the corresponding samples and clusters, and highlighting haplotypes previously reported from other countries, annotated with their known geographical distribution.

Table 11. Haplotypes of the Austrian isolates, their corresponding clusters and the countries in which these haplotypes have been previously reported, based on reference sequences used in this study. Haplotypes not shared with any reference sequences are indicated with "no reference" (**n.r.**). Isolates with travel history are marked: Indonesia, Thailand, Singapore (▼); Indonesia (▲); Bulgaria (★); no travel history (■). Unmarked isolates have missing travel information.

Haplotype	Austrian isolates	Cluster	Known distribution
cox1			
cH5	10A1	A	Brazil China Thailand
cH21	16A1▼, 19A1▲, 23A1, 24A2		Brazil Thailand
cH37	18A2■	C	n.r.
cH38	10A2		n.r.
cH39	24A1★		n.r.
cH40	18A1		n.r.
nad1			
nH37	16A1▼	A	n.r.
nH38	10A1		n.r.
nH39	24A2		n.r.
nH40	24A1★	C	n.r.
nH41	18A1		n.r.
nH42	19A1▲	A	n.r.
nH43	23A1		n.r.
ITS1			
iH1	18A1	B	China Laos Myanmar Thailand
iH13	10A1	A	Bangladesh Brazil China Indonesia Iraq Japan Korea Laos Myanmar Thailand
iH18	23A1		n.r.
iH19	16A1▼, 18A2■, 19A1▲	B	n.r.
iH20	24A1★, 24A2		n.r.
iH21	10A2		n.r.

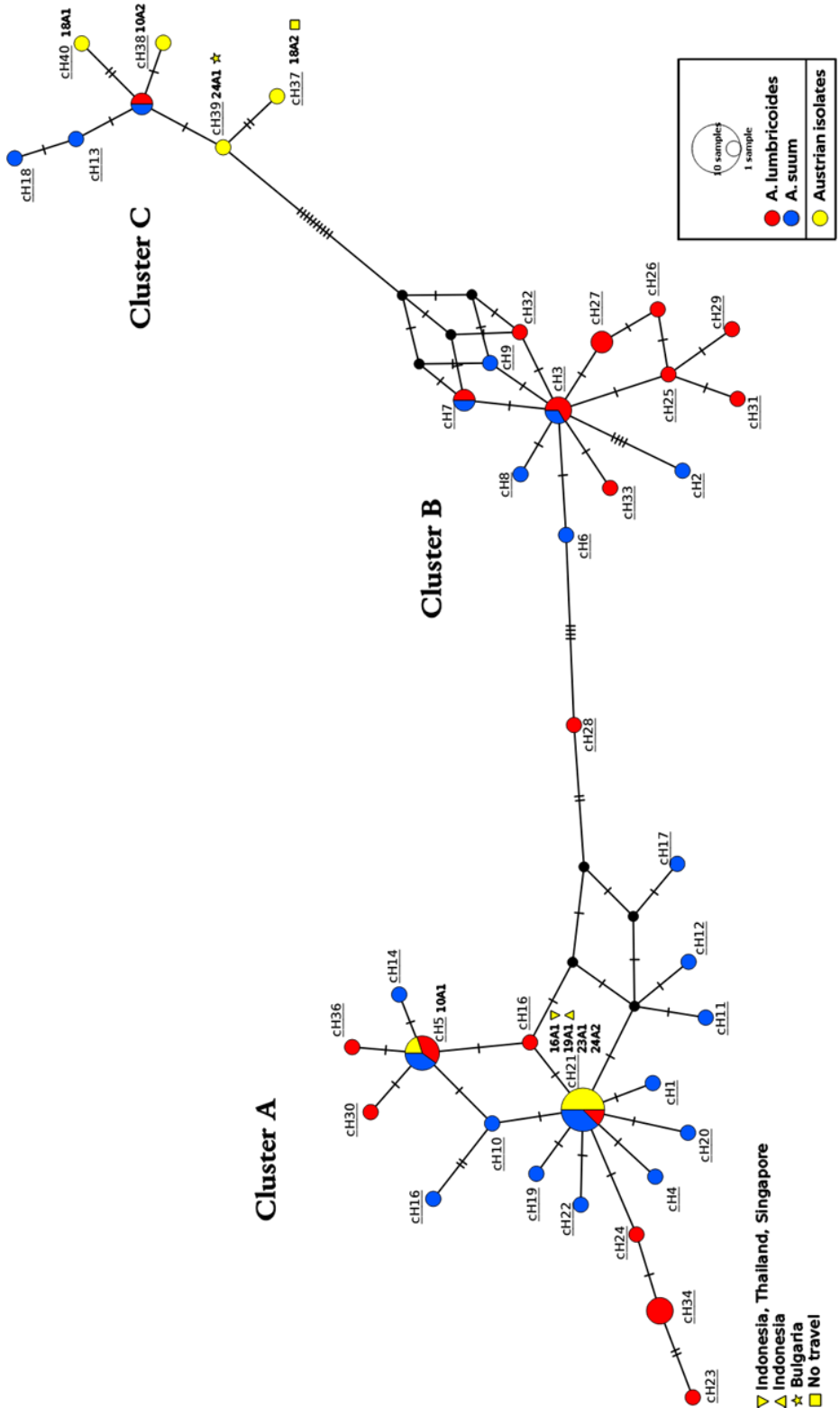

Figure 15. Median joining network of *cox1* sequences of human Ascaris spp. isolates from Austria (n=9) and reference sequences (n=50). Each circle represents a unique *cox1* haplotype (cH) and the circle size is proportional to the haplotype frequency of all included sequences. Colors represent species of the reference sequences (red, *A. lumbricoides*; blue, *A. suum*) and Austrian isolates (yellow) present in each haplotype. Sample names of the Austrian isolates are marked in bold next to their associated haplotypes, including information on travel history: Indonesia, Thailand, Singapore (▼); Indonesia (▲); Bulgaria (★); no travel history (■). Unmarked isolates have missing travel information. Black circles denote inferred intermediate haplotypes. Bars indicate single nucleotide differences. Haplotypes group into three clusters (A, B, C) (created via PopART).

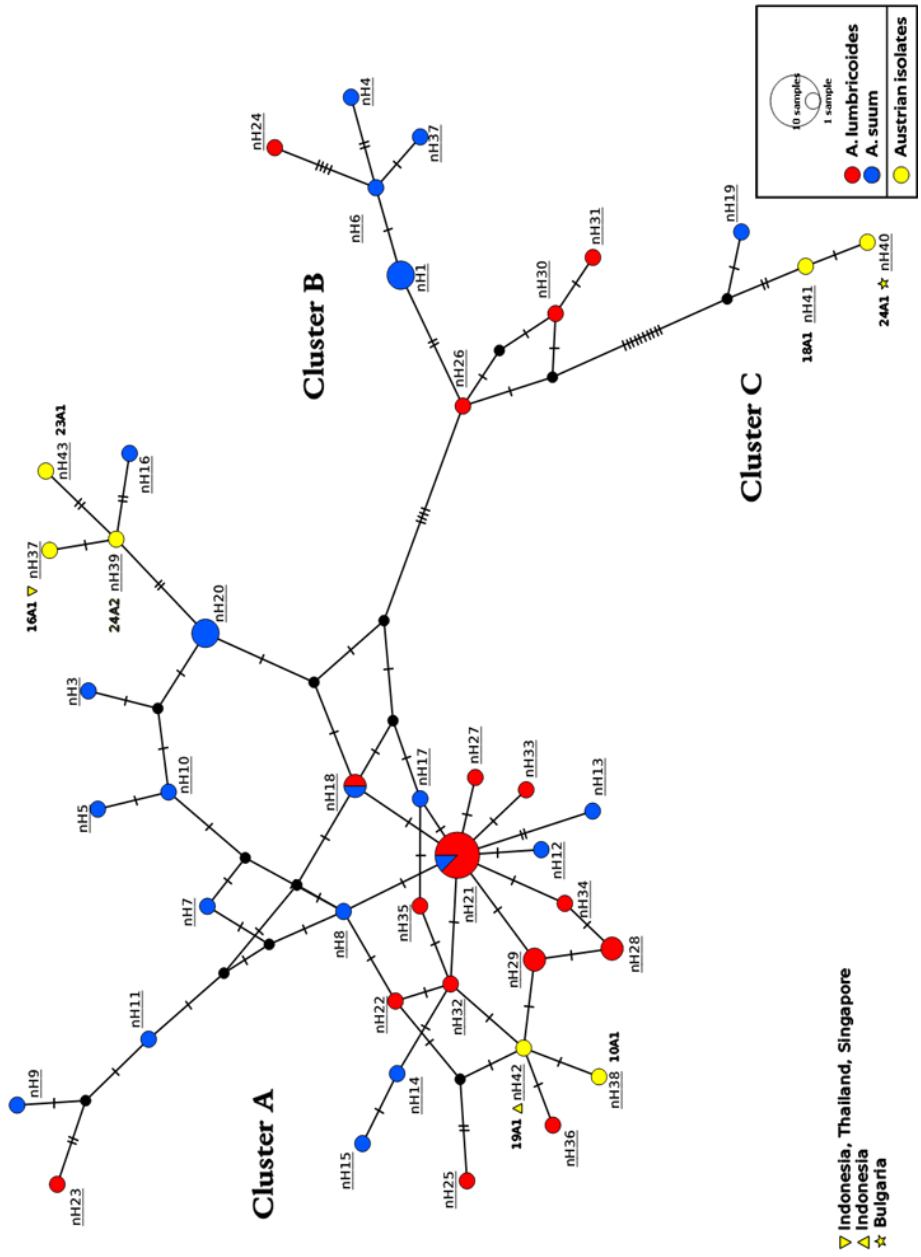

Figure 16. Median joining network of *nad1* sequences of human *Ascaris* spp. isolates from Austria (n=7) and reference sequences (n=50). Each circle represents a unique *nad1* haplotype (nH) and the circle size is proportional to the haplotype frequency of all included sequences. Colors represent species of the reference sequences (red, *A. lumbricoides*; blue, *A. suum*) and Austrian isolates (yellow) found in a haplotype. Sample names of the Austrian isolates are marked in bold next to their associated haplotypes, including information on travel history: Indonesia, Thailand, Singapore (▼); Indonesia (▲); Bulgaria (★). Unmarked isolates have missing travel information. Black circles denote inferred intermediate haplotypes. Bars indicate single nucleotide differences. Haplotypes group into three clusters (A, B, C) (created via PopART).

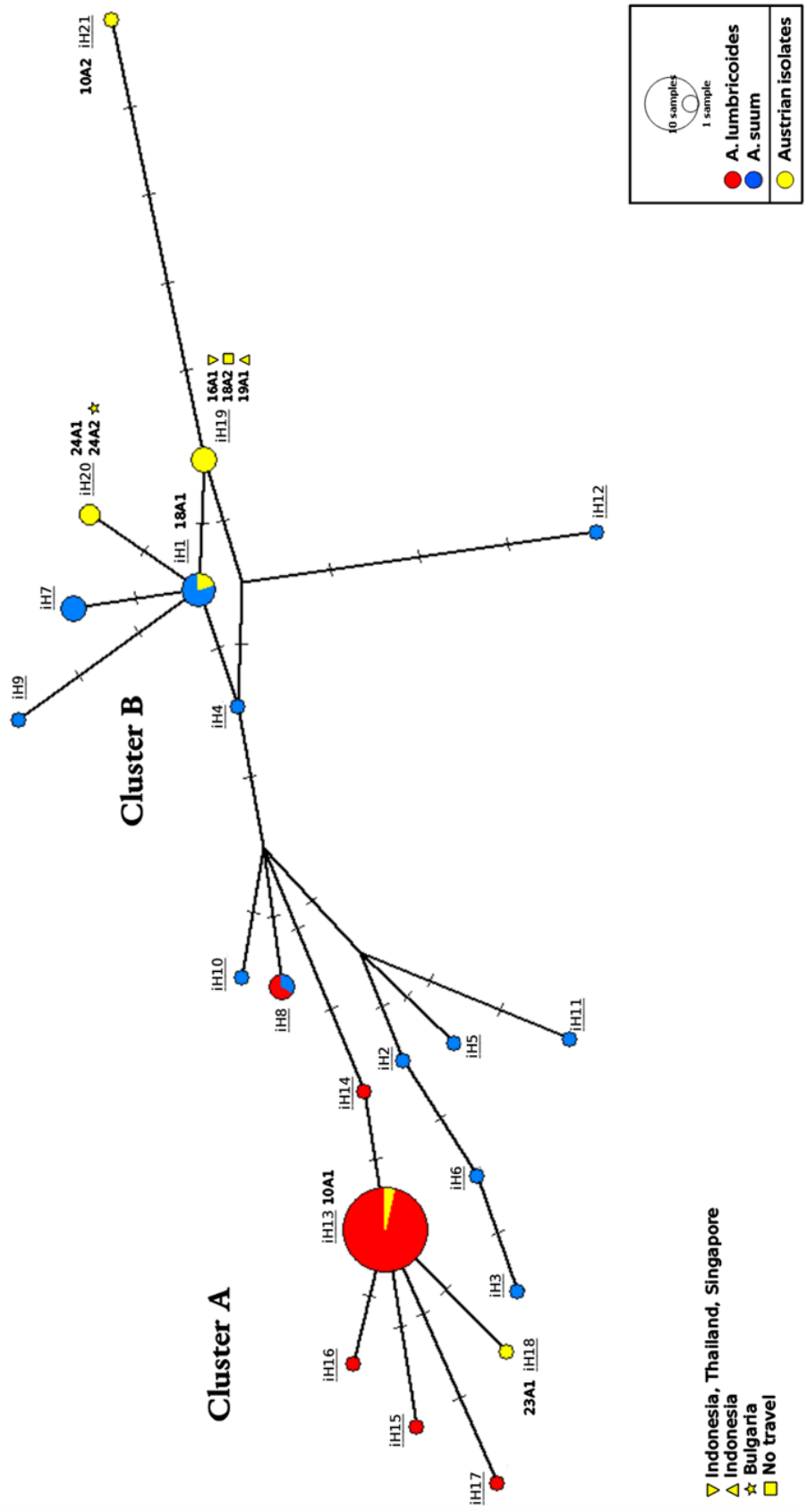

Figure 17. Median joining network of ITS1 sequences of *Ascaris* spp. isolates from Austria (n=9) and reference sequences (n=50). Each circle represents a unique ITS1 haplotype (iH) and the circle size is proportional to the haplotype frequency of all included sequences. Colors represent species of the reference sequences (red, *A. lumbricoides*; blue, *A. suum*) and Austrian isolates (yellow) present in each haplotype. Sample names of the Austrian isolates are marked in bold next to their associated haplotype, including information on travel history: Indonesia, Thailand, Singapore (▼); Indonesia (▲); Bulgaria (★); no travel history (■). Unmarked isolates have missing travel information. Forks without circles mark inferred intermediate haplotypes. Bars mark single nucleotide differences. Haplotypes primarily accumulated in 2 clusters (A, B) (created with Fluxus Engineering Network (fluxus-engineering.com)).

4. Discussion

This study represents the first molecular phylogenetic analysis of *Ascaris* isolates from human cases in Austria, a country since several decades considered free of endemic ascariasis. The aim was to reveal the genetic diversity of *Ascaris* isolates collected over the past 14 years and to assess the transmission dynamics of ascariasis in Austria. In particular we aimed to find out if *A. suum* is a possible causative agent of human ascariasis in Austria and to which species *Ascaris* isolates from patients without travel history belong to. The comparative analyses of the mitochondrial cytochrome C oxidase 1 (*cox1*) and NADH dehydrogenase subunit 1 (*nad1*) gene sequences, as well as the nuclear internal transcribed spacer 1 (ITS1) region, using the nine Austrian isolates and 50 reference sequences, including 83 *A. lumbricoides* and 67 *A. suum* sequences, revealed comparably high genetic diversity in the Austrian isolates, which varied depending on the genetic region analyzed. In all three analyses, the isolates grouped into two different clusters, but with varying haplotype clusters, highlighting the complexity of *Ascaris* population structure. The mitochondrial *cox1* and *nad1* networks did not allow a clear species-level separation, as both clusters contained isolates identified as *A. lumbricoides* and *A. suum*. In contrast, a rough differentiation between the two taxa was possible based on the ribosomal ITS1 marker.

This study corroborates the hypothesis that hybrids between *A. lumbricoides* and *A. suum* exist and that a clear separation of the two species is not possible. Altogether, the results support that *A. lumbricoides* and *A. suum* are part of a species complex, rather than two separated species.

4.1 Genetic markers

Due to the difficulties distinguishing between *Ascaris* species morphologically (Monteiro et al., 2019), numerous PCR protocols targeting different molecular markers have been developed over the past 20 years (Zhou et al., 2020), with polymorphic markers *cox1*, *nad1* and ITS1 being the most used targets, beside microsatellite markers (Cavallero et al., 2013).

Of the different genetic markers used in this study, *cox1*, using the PCR protocol of Peng et al. (2005), gave the most robust results with clear bands and a low sequencing error rate.

In contrast to *cox1*, amplification of the *nad1* gene according to Peng et al. (2005) proved to be more error-prone and required adjustments to the amplification conditions. Amplification of the *nad1* gene using the original cycling conditions produced no results. Several factors can influence the success of amplification, but no evident reason could be identified. Insufficient DNA input seems unlikely, as amplification was performed several times with differing amounts of template DNA. Similarly, faulty primers and incorrect concentrations or compositions of PCR components can be ruled out due to the successful amplifications of other isolates. Partial DNA degradation is considered improbable, since *cox1* and ITS1 genes were successfully amplified from these isolates, indicating intact and amplifiable DNA. However, the complete absence of band formation in gel electrophoresis for the unsuccessful isolates indicates that no amplification, not even partial, occurred. Whether this was caused by inappropriate DNA cycler conditions or human error during sample preparation remains uncertain (Bio-Rad Laboratories, n.d.).

Amplification of the nuclear ITS1 region, according to Ishiwata et al. (2004), also proved to be more error-prone and required the usage of additional primers. Primer slippage is assumed to be the most likely cause of this amplification error. Primer slippage refers to the misalignment of a primer during the elongation process, which results in a shift of the primer position on the template and, consequently, to amplicons of varying length and quality. This is known to occur in homopolymeric regions with low sequence complexity (Elbrecht et al., 2018; Microsynth, n.d.), which is consistent with the T rich segment identified in the ITS1 region, between positions 110 and 128 in the analyzed sequences.

Nevertheless, both the *nad1* protocol described by Peng et al. (2005) and the ITS1 protocol described by Ishiwata et al. (2004) have been widely cited and successfully applied in numerous studies (Betson et al., 2014; Boeira et al., 2025; Liu et al., 2012; Roepstorff et al., 2011; Sadaow et al., 2018), underscoring their general robustness and reliability.

4.2 Genetic diversity of *Ascaris* isolates

A total of nine *Ascaris* samples, isolated from humans in Austria, were examined for their *cox1*, *nad1* and ITS1 genes, which were then compared with reference strains retrieved from the GenBank database. A total of six *cox1*, seven *nad1*, and six ITS1 haplotypes were identified. Of these, two are novel *cox1* haplotypes, seven novel *nad1* haplotypes and four novel ITS1 haplotypes, that have not been reported previously.

Cox1 haplotype cH21 comprised four Austrian isolates (samples 16A1, 19A1, 23A1 and 24A2) sharing 100% (383bp/383bp) sequence identity with reference sequences from Brazil and Thailand. Haplotype cH5, containing sample 10A1, matched sequence identity with references from Brazil, China and Thailand. Comparisons with sequences available on the GenBank database showed that sample 18A2 of haplotype cH37 shared sequence identity with an *Ascaris* genome reported from Germany (OZ376524) and sequences from Brazil (MK143379). Likewise, sample 24A1, representing haplotype cH39, also matched a genome originating from Germany (OZ376547). These genomes were not included as reference sequences in this study. The remaining *cox1* haplotypes identified were unique to our Austrian isolates and have not been reported before.

Similarly, all seven *nad1* haplotypes identified in this study proved to be unique and could not be assigned to any known haplotypes, neither in comparison with the selected references, nor via GenBank comparison, but showed high similarity (98.8%-99.7%) with sequences from Brazil (HM602028.1, MK160568.1), Germany (OZ376547.1, OZ376552.1), Kenya (OZ376595.1) and Thailand (OZ376561.1).

The ITS1 analysis revealed, as with *cox1*, known and unique haplotypes among the Austrian isolates. ITS1 haplotype iH1 was identified in sample 18A1 and iH13 in 10A1, both showing global distribution, with reports from Bangladesh, Brazil, China, Indonesia, Iraq, Japan, Korea, Laos, Myanmar and Thailand. In contrast, haplotypes iH18 and iH21 were each identified in single Austrian isolates (samples 23A1 and 10A2 respectively), while haplotype iH19 comprised of samples 16A1, 18A2 and 19A1, and haplotype iH20 included samples 24A1 and 24A2. These haplotypes could not be assigned to any previously published haplotypes but showed the highest sequence identity (>99%) to sequences from China (KY964445.1), Indonesia (OQ539679.1) and Thailand (MF358963.1, PP758228.1).

This level of haplotype variability, despite the relatively small sample size, indicates a considerate genetic heterogeneity of Austrian *Ascaris* isolates. This is underscored by the fact that only three of the identified haplotypes are shared by multiple isolates (cH21, iH19, iH20). Comparable levels of haplotype diversity have been reported in other studies within Europe (Cavallero et al., 2021), but also geographically more distant regions, such as Uganda (Betson et al., 2012) or India (Das et al., 2015). A similar pattern was, for example, reported by Iñiguez et al. (2012) in Brazil, where analyses based on *cox1* and *nad1*, revealed a mixture of novel, unreported haplotypes and widely distributed haplotypes, known from

regions such as Angola and China. Likewise, Leles et al. (2010) documented high genetic diversity in Brazilian *Ascaris* populations using ITS1 as genetic marker. The genetic diversity of *Ascaris* found in Austria is therefore comparable to that of other countries.

However, the apparent extent of genetic diversity is influenced by both the number of isolates examined and the number of reference sequences used in this study. To enhance the validity of our analyses, reference sequences were selected with some degree of randomness, while aiming to capture a broad geographic representation. Sequences shorter than ours were not included, to avoid bias in the alignment and to facilitate comparability.

4.3 Haplotype network analysis and inference on species separation

The haplotype networks for *cox1* and *nad1* both showed a three-cluster formation, with clusters containing *A. lumbricoides*, *A. suum*, as well as our Austrian isolates, without a clear separation between species. This pattern has been previously described in earlier phylogenetic studies, as in Cavallero et al. (2013), Monteiro et al. (2019) and Sadaow et al. (2018), targeting the same genes. Both networks share that Austrian isolates group into clusters A and C and are absent from cluster B. The haplotypes in cluster B showed a wide geographic distribution. In the *cox1* network, it comprised sequences from Brazil, China, Denmark, Japan, Laos and Uganda, whereas in the *nad1* network it included sequences from Brazil and China. Therefore, the haplotypes did not show any clear segregation based on geographic location. However, none of the Austrian isolates were associated with this cluster.

In the *cox1* network, four of our isolates clustered within haplotype cH21, representing the most frequent haplotype, including two isolates with documented travel history to Indonesia, all assigned to cluster A. Comparative sequences within this haplotype (n=4) originated from Brazil and Thailand. Haplotype cH5, containing sample 10A1, likewise was located within cluster A. The remaining four *cox1* haplotypes identified in this study were grouped within cluster C and included both isolates from a patient with travel history to Bulgaria (24A1) and one without any recorded travel history (18A2). Cluster A showed the highest concentration of haplotypes, followed by cluster B and then C.

The *nad1* network showed that Austrian isolates were dispersed across seven distinct haplotypes across cluster A and C. The most frequent haplotype, nH21, included none of

the Austrian isolates. Those with documented travel history were dispersed across the haplotype network. Isolates from patients with travel history to Indonesia were assigned to different haplotypes, namely nH37 (16A1) and nH42 (19A1), both located within cluster A. In contrast, sample 24A1, for which no travel history was recorded, was assigned to its own haplotype, nH40, within the same cluster. Cluster C contains only three haplotypes (nH19, nH40, nH41), all based on one sequence each, one reference sequence and two isolate sequences. Unlike clusters B and C, cluster A contained a significantly higher number of haplotypes.

Contrary to the other networks, the ITS1 network showed the formation into two clusters, A and B, with two outlier haplotypes (iH12 and iH21) connected to cluster B. Cluster A comprised haplotypes iH13 and iH18 (containing isolates 10A1 and 23A1 respectively), with iH13 representing the most frequent haplotype. The remaining haplotypes were grouped within cluster B and included Austrian isolates with travel history to Indonesia (samples 16A1 and 19A1), an isolate without recorded travel history (sample 18A2), all assigned to haplotype iH1, as well as sample 24A1 with travel history to Bulgaria, which was assigned to haplotype iH20.

The ITS1 alignment and its corresponding haplotype network allowed species separation, in contrast to the mitochondrial markers. Nucleotide variation at positions 5, 6, 130 and 244 have been proposed as discriminatory sites for separating *A. lumbricoides* and *A. suum* by Sadaow et al. (2018). According to this scheme, isolates 10A1 and 23A1 may be identified as *A. lumbricoides* and the remaining seven samples as *A. suum*, following sequence alignment. This result was supported by the generated network, which showed a separation between cluster A, dominated by *A. lumbricoides*, and cluster B, dominated by *A. suum*. Therefore, the placement of isolates 10A1 and 23A1 in cluster A would indicate two cases of autochthonous infestation with *A. lumbricoides* in Austria. Among the seven isolates assigned to *A. suum*, three isolates (16A1, 19A1, 24A1) have reported travel activities abroad, suggesting that these infestations may have been imported. In contrast, isolate 18A2, with no travel activity, would imply at least one autochthonous infection with *A. suum* in Austria. However, the lack of recorded travel histories for five of the isolates, including our *A. lumbricoides* associated samples 10A1 and 23A1, does not allow further implications.

However, the reliability of ITS1 sequences as sole discriminator between *Ascaris* species remains controversial, due to the high intra-host variability of isolates, which has been

observed in a study be Leles et al. (2010). It is advised to interpret species identification based on a single marker with caution (Anderson, 2001). Nevertheless, markers *cox1* and *nad1* have proven equally problematic, as the heterogeneous species distribution in the respective network makes it impossible to distinguish between species. This was also reported by Monteiro et al. (2019) and Nejsum et al. (2017), who assessed the genetic diversity of *Ascaris* using *cox1* and *nad1* sequence data. This raises the question if hybrid species are involved in this study. Since clusters in our mitochondrial networks contain multiple haplotypes with different species association, a distinct hybrid cluster cannot be defined. This supports the interpretation of *Ascaris* as a species complex with ongoing gene flow (Nejsum et al., 2017). Due to the limitations of *cox1*, *nad1* and ITS1 as genetic markers, the usage of microsatellites has been suggested for further studies. Their higher mutation rates, compared to ribosomal markers, provides better resolution for distinguishing closely related taxa and may help to differentiate between *Ascaris* species and to uncover hybrid lineages (Betson et al., 2014; Nejsum et al., 2017).

Altogether, the results reveal a high level of genetic diversity among *Ascaris* strains involved in human cases of ascariasis in Austria. The lack of clear species-specific clustering in the mitochondrial data sets supports the hypothesis that *A. lumbricoides* and *A. suum* may in fact not be two different species, but rather a single, highly interbred *A. lumbricoides*/*suum* species complex, as suggested by Leles et al. (2012) and Cavallero et al. (2013).

4.4 Implications on transmission dynamics and zoonotic potential

Although a clear discrimination of species was not possible, the genetic patterns observed in this study have significant implications: although all *Ascaris* isolates have human origin, they show considerable genetic diversity between them and seven isolates clearly cluster with *A. suum*. Several studies have shown evidence that cross-transmission occurs between the two presumed species (Cavallero et al., 2013; Jesudos Chelladurai et al., 2017; Sadaow et al., 2018), that hybridization occurs (Criscione et al., 2007; Easton et al., 2020; Zhou et al., 2012), that both *A. lumbricoides* (Leles et al., 2012) and *A. suum* (da Silva et al., 2021), can complete their life cycles in both hosts and that some genotypes are common in both species (Peng et al., 2007), supporting this hypothesis (Alves et al., 2016). The concept of a species complex rather than two individual species has implications for understanding the

transmission dynamics of *Ascaris*, particularly in countries with intensive pig farming, including Austria.

From a public health perspective, this means that *Ascaris* infections can not only be imported through international travel but are also zoonotic via contact with infested animals, contaminated soil or food. Several studies have confirmed the potential of pig-derived ascariasis in humans (Arizono et al., 2010; Silva et al., 2021). In countries with intensive pig farming where pig feces are commonly used as fertilizers, such as Austria, the potential for zoonotic transmission should not be underestimated. According to Statistics Austria (2025), Austria is home to roughly 16,600 pig farms housing a total of approximately 2.5 million pigs, bearing a considerable zoonotic potential for human *Ascaris* infestation. As previously described, Joachim (2006) reported in a survey that signs of infection with *A. suum* were detected in 19% of pigs on 15 out of 18 farms, implying a high *A. suum* prevalence in Austria. However, high infection rates have also been reported from several other European countries. In Italy, 92.2% of 399 investigated farms were tested positive for *A. suum*, and the characteristic “milk spots”, indicative of larval migration, were observed in 26.4% of 754,833 examined pig carcasses. (Allievi et al., 2025). In Ireland, an infection rate of 30% was observed across 20 farms (Senanayake et al., 2025). In Poland, 28.6% of pigs were tested positive for *Ascaris* eggs in a survey covering 70 farms (Kochanowski et al., 2017). In the Netherlands, infections were detected on 50% of 36 investigated farms (Eijck & Borgsteede, 2005), while in Denmark all five examined farms were tested positive for *A. suum* (Katakam et al., 2016). This prevalence is not restricted to Europe and has also been reported across Asia and Africa. In Asia, 65.5% of 5,511 fecal samples from pigs were tested positive for *A. suum* eggs in Nagaland, India (Laha et al., 2015), 45% of pigs across 18 farms were infected in Nepal (Adhikari et al., 2021) and 36.7% prevalence was reported in pigs in China (Boes et al., 2000). In Africa, infection rates of 44.5% were reported from 77 pig samples in South Africa (Fourie et al., 2019) and 40% of 383 pig samples were tested positive in Burkina Faso (Tamboura et al., 2006).

In regions with intensive pig production, the application of manure to agricultural land may lead to persistent contamination of the environment as *Ascaris* eggs can survive in moist soil for several years. The risk of spreading is increased by the fact that their sticky, proteinaceous shell enables them to stick to a wide range of surfaces, such as vehicles, equipment, insects and animal skin, which allows further mechanical spread (Joachim,

2006). The zoonotic potential has been reported from other countries considered non-endemic for ascariasis as well, including Japan (Arizono et al., 2010), the United States (Chelladurai et al., 2017) and the UK (Bendall et al., 2011), where low levels of human *Ascaris* infestations occurred. In all these studies, genotype analyses revealed that the *Ascaris* isolates detected in humans were genetically more consistent with lineages that are predominantly found in pigs, supporting a zoonotic origin of the infestation. Additionally, risk factor analyses were performed in the study of Bendall et al. (2011), that linked cases of ascariasis in humans living in close proximity to pig farms to zoonotic transmission. The findings of the current study therefore mirror a global pattern where *Ascaris* populations circulate between humans and pigs, while maintaining a local reservoir, even in areas with good sanitation and segregated human and pig populations.

However, an additional zoonotic reservoir must be considered besides domestic pig farming. Wild animals represent a potential reservoir for *Ascaris* in Austria. Auer (2011) reported on the presence of *Ascaris* infestation in wild boar populations and describes the risk of infestation from hunted game. Similarly, Matsumura et al. (2025) reported *Ascaris* infestation in wild boars in Japan, yet identified the infecting species as *A. lumbricoides* instead of *A. suum*, based on ITS1 identification. Furthermore, it has been demonstrated that *A. lumbricoides* also infects primates, such as orangutans (Civáňová Křížová et al., 2023), indicating a broader host range and potentially additional zoonotic transmission pathways beyond those previously recognized.

In this study, three isolates were included from patients with confirmed travel history, with two patients having traveled to regions in south-eastern Asia and one to Bulgaria, regions where ascariasis remains endemic (Agustina et al., 2023; Harizanov et al., 2020). These cases suggest that imported infestations continue to contribute to the occurrence of human ascariasis in Austria and must be considered alongside autochthonous zoonotic infection. According to Statistics Austria (2024), 14.5 million (52.7%) of the 27.53 million vacation trips taken by the Austrian population in 2024 were to destinations abroad. The most popular destinations were Italy (19.8%), Germany (14.7%), Croatia (11.8%), Spain (5.7%), Greece (4.2%), Hungary (3.9%), Turkey (3%), the Czech Republic (2.9%), France (2.7%) and the United Kingdom (2.1%). Long-distance travels outside Europe and Turkey accounted for 7.9% of all trips. South-eastern Asia, including Indonesia, Laos, Malaysia, Myanmar, the Philippines, Sri Lanka and Vietnam, has one of the highest prevalences (19.06%) of *Ascaris* infections worldwide (Holland et al., 2022). While Central Europe is

considered non-endemic, ascariasis is one of the most common helminth infections in Eastern Europe and the Balkans. According to a study by Hotez & Gurwith (2011), the prevalence of ascariasis in adults and children from Albania was 1%, from Armenia 4%, from Turkey 69%, and in children from Poland 1%. However, studies from European countries assumed non-endemic for ascariasis have demonstrated local hybridization and cross-infection between *A. lumbricoides* and *A. suum*, including Italy, Hungary and Slovakia (Cavallero et al., 2013), as well as zoonotic transmission in Denmark (Nejsum et al., 2005). The high volume of travel between Austria and neighboring countries, such as Italy, as well as regions with high *Ascaris* prevalence, including Turkey and South-eastern Asia, is therefore likely to contribute to the genetic diversity observed among Austrian isolates. In this context, Visiting Friends and Relatives (VFR) travel, defined as trips undertaken by migrants or their descendants to visit family and friends in their countries of origin (Shaughnessy et al., 2025), warrants particular attention. Such travel within migrant communities may substantially influence the introduction and circulation of infections when visits occur to endemic regions. According to the Austrian Integration Fund (2024), as of January 1, 2024, approximately 165,300 people born in Turkey and 124,100 Turkish citizens were living in Austria, making them the third-largest group of foreign nationals after those from Germany and Romania. Regular travel between Austria and Turkey within this community may therefore represent one potential pathway for the introduction of *Ascaris* infections and similar dynamics may apply to other migrant populations. However, an accurate assessment of the impact of travel activities is hampered by a lack of patients travel data. Patients sometimes forget to mention travel activities or stays abroad are not perceived as such, especially short stays or stays in non-tropical regions.

Nevertheless, this study shows considerable haplotype diversity in Austrian Ascaris isolates, which likely arises from a combination of imported infections and autochthonous transmission. Given the intensive pig farming in Austria and the occurrence of *Ascaris* in both domestic pigs and wild animals, such as wild boars, zoonotic transmission represents a plausible and relevant route of infection. The observed number and distribution of haplotypes, however, represent only a snapshot in time and may change through genetic exchange between *Ascaris* populations, mutation and introduction of new haplotypes.

4.5 Study limitations and outlook

While this study provides valuable insights into the diversity of *Ascaris* causing human infestations in Austria, certain limitations must be acknowledged. Due to the lack of legal regulations in Austria requiring the reporting of human ascariasis cases and the limited number of cases, only a small number of isolates were available. Additionally, essential information, such as patients' recent travel history, was missing from five medical records or might be potentially incorrect due to patients not reporting all their travels, leading to the lack of potentially relevant epidemiological information.

This study supports the need for a One Health approach. Sharing data between physicians and veterinarians, and the sharing of information with research institutes, may provide deeper insights and more reliable data. In future studies, the usage of microsatellites should be considered, and broader sampling should be extended also to pigs to enable haplotype comparison to be carried out for isolates from both hosts.

Overall, the results of this study support an epidemiological model in which imported infections and zoonotic transmission contribute to the *Ascaris* situation in Austria, with pigs as a steady reservoir. Although only a small number of samples were analyzed, a high level of genetic diversity was found.

5. Conclusions

This study provides first insights into the molecular diversity of Austrian *Ascaris* isolates from human patients with and without travel history. The results revealed a high genetic diversity among Austrian isolates, with six *cox1*, seven *nad1*, and six ITS1 haplotypes identified across nine samples. While the ITS1 marker allowed two isolates to be identified as *A. lumbricoides* and seven as *A. suum*, our results also showed that a clear differentiation of *A. lumbricoides* and *A. suum* is not possible. This study altogether supports the concept of a genetically divers *A. lumbricoides/suum* species complex and highlights that ascariasis in non-endemic countries should not be regarded solely as an imported disease.

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