CHAPTER 4

Polytene Chromosomes

Dipterans endowed with polytene Chromosomes are the store house for cytological investigation that leads to their proper taxonomic status. Presence of polytene chromosomes in dipterans is rather a monopoly in the animal kingdom. Polytene chromosomes are the product of endomitosis where the chromatin treads divide again and again within the cell with the nuclear membrane intact. Each nuclei bear haploid set of chromosomes with paired parallel interphase chromatids of the diplod individual. Polytene chromosomes are best found for studies in those tissues having secretory function such as Salivary gland, Malpighian tubules and mid gut, rectal papillae. The polytene chromosomes from the salivary gland nuclei of mature larvae offer the best resolution for studies in comparison to pupa and adult. However, the banding pattern of the polytene chromosomes is conserved in all the stages. The degree of polytenization depends on the physiological conditions of the tissue from where the chromosomes are obtained. The quality of polytene chromosomes has been observed to be affected by biological and ecological factors such as seasonal variation along with other limiting factors.

In Simuliids, existence of sibling is rather a rule which is why the cytological investigation of the polytene Chromosomes becomes inevitable.

4.1 Cytogenetics

Cytogenetic studies offer the best way to understand the population genetics. Among the Dipteran flies Drosophiliids have been best studied for genetic analysis through laboratory reared populations. The resolution of the Simuliid polytene chromosomes excel over the Mosquitoes and Drosophiliid chromosomes but due the challenges of providing suitable environmental conditions in the laboratory make it very difficult to maintain a Simuliid strain in laboratory as the Simuliids lay eggs in the running water where the larval and pupal development takes place to emerge as an adult. The adult female black flies are haematophagus. However, Edman and Simmons, 1985, have successfully reared few species of black flies overcoming the challenges demanded by the flies for laying of eggs in flowing water and other developmental conditionings.

The chromosomes of all the species of black flies of Darjeeling region so far studied have n=3, which is similar to the species of Simuliids reported from other parts of the world. In case of other Simuliids such as in *Cnephia* and in the members of the sub genus *Eusimulium* the Chromosome number have been reduced to n=2. The species of *Simulium* investigated from Darjeeling have shown

bear Metacentric and Submetacentric Chromosomes. Most of the species studied have shown to be reproductively conservative.

Records of cytogenetic studies of the black flies are scattered and scanty in comparison to other groups of organisms. Only about 28 % of the total population of the black flies deals with the cytogenetic studies around the world.

Polytene chromosome analysis of Simuliids serves the purpose of studying the frequency of certain phenomena at both population and species levels. Polytene chromosome study can clearly differentiate between the sibling species and polymorphism and add to the morphotaxonomic status of the fly.

4.2 Chromosomal Complement

Almost all the black flies around the world predominantly have haploid number of 3 chromosomes excluding some other species of black flies such as *Cnephia pallipes* of the northern Palearctic region (Procunier, 1982b) , and all the members of subgenus *Eusimulium* where the haploid set of chromosomes have been reduced to n=2 (Leonhardt, 1985). This is in all probability due to the fusion of two of the three chromosomes. In addition to normal haploid set of chromosomes, additional or supernumerary chromosomes or 'B' chromosomes have also been reported in Simuliids.

Most of the black flies are diploid but the presence of Triploid individuals is also reported. Eight such species have been recorded of which four of them are recorded from North America. These species are parthenogenetic and can be of autotriploid or allotriploid in origin. *Stegoutate mutata* is probably of autotriploid origin (Basrur and Rothfels 1959), *Gymnopais dichopticoides, G. holopticoides, Prosimulium ursinum* are allotriploids, derived from two different parental species (Rothfels 1979).

4.3 Chromosomal Rearrangements

Chromosomal rearrangement is one of the most important phenomena that play an important role in the study of Cytotaxonomy of black flies may pave ways for the speciation. This phenomenon is abundant in the Simuliid family. There are different types of chromosomal rearrangements occurring in chromosomes, which are clearly visible and identifiable in polytene chromosomes. Among the rearrangement of chromosomes inversions are the most common ones. The rearrangement of the section due to inversions may appear at any location of the chromosome. It can occur both in autosomes and sex chromosomes. Even a single inversion can bring about significant changes in due course of time in evolution. They may be continue to exist in one line of generations and may be lost in other line. Some rearrangements involve the centromere and called Pericentric inversions which account for 3% of the total inversions and 97% are paracentric inversions. The inversion that does not involve centromere but involves only chromosomal arms is called

paracentric inversion. Paracentric inversions are more common in comparison to Pericentric inversion. Chromosomal inversions are thought to suppress crossing over.

The inversions may be interspecific or fixed inversions or monomorphic which do not occur heterozygously and intraspecific which occurs heterozygously is called floating inversions which are polymorphic.

In addition to inversions other type of rearrangements takes place such as deletions, insertions, duplications, band dimorphisms, translocations, and chromocenters.

Occurrence of both the phenomena of deletion and inversions of individual bands or sections are not frequent. *Simulium innoxium* provide one example of such rearrangement where deletion of a small section from the base of the short arm of the second chromosome and its reinsertion in a similar position in the short arm of the third chromosome (Bedo 1975a; as *S. pictipes* "B").

In *Twinnia hirticornis* a heterozygous deletion of the nucleolar organizer from the centromere region of the first chromosome take place which subsequently is reinserted in the short arm of the same chromosome (Rothfels and Freeman 1966; as *T. nova*)

The phenomenon of duplication of bands in case of Simuliidae has not been documented.

On the other hand, quite common type of rearrangement is the band dimorphism in Simuliidae especially in conditions where bands occur in the heterozygous condition (Bedo, 1975a; Rothfels and Featherston, 1981).

Translocations are also not very frequent. In case of *Simulium woodi* in Africa a mid – arm translocation has been discovered (Procunier and Muro 1994). In 28 North American taxa the whole arm interchanges have been documented: *Twinnia nova, T. hirticornis, Heldon vernalis, the 12 species of the subgenus Prosiumulium transbrachiuym, Heldon,* also in the four eastern species of the *Prosimulium magnum* species group, the seven nominal species I the genus *Metacnephia,* and *Simulium decimatum* (Ottonen 1966, Rothfels 1979, Procunier 1982a, Rothfels and Freeman 1983, Shields 1990).

Centromeres in Simuliids exhibit in both forms in an individual chromosome or the centromeres of all the polytene chromosomes fuse to form a chromocenters. Ectopic pairing, association of the Centromeres, the pseudo-chromocenters, which resemble a true chromocenters but typically does not occur in all nuclei of the larval silk glands or in all population (Rothfels and Freeman, 1977) are also not uncommon. In the species where the chromocenters are

present, the chromosomal arms radiate from a central heterochromatic mass of varying size are an important tool for species-specific diagnostics. The chromocenters are a constant feature of a polytene complement, as in case of Drosophiliids, occurring infrequently as polymorphism (Brockhouse et al., 1989). Several species of North America like *Prosimulium fulvum*, *P. Shewelli* are polymorphic for a chromocenters. The size of the chromocenters is variable. The cytoform *Simulium praelargum* "IIIL-1.2" from Darjeeling, West Bengal, India also exhibits a large enhanced chromocenters in relations to its counterpart species, *Simulium praelargum*-st and *Simulium praelargum "*IL" (Henry et al., 2010; Thapa et al., 2014). In some cases it is so large that it can be visualized even in a normal somatic nucleus as in case of *Simulium chrimatinum* n. sp. and *Simulium chromocentrum* n. sp. In case of a partial Chromocenters that involve only two of the three chromosomes is known in *Simulium croxtoni* (Hunter and Connolly 1986), *Prosimulium imposter* (some Arizona populations only) and *Simulium ghoomense* (Henry et al., 2010).

Sex chromosomes also exhibit rearrangements which are very important. Male black flies as in humans are heterogametic (XY) and females are homogametic (XX). However, in few taxa, females are heterogametic such as one species in Equador (Procunier et al., 1987) and Japan (Hadi et al., 1995), and the Polynesian subgenus, *Inseliellum* (Rothfels 1989). Of the three chromosomes any one chromosome can serve as the sex chromosome. In some cases such

as in the Darjeeling Himalayan black flies, the simple form of sex chromosomes are not distinguished morphological in a typical microscopic preparation. These types of undifferentiated sex chromosomes are presumed to be ancestral condition (XoYo) within a population which is present in half of the 155 North American species (Adler et al., 2004). Cytologically differentiated sex chromosomes (X1Y1, X2Y2) bearing species are also prevalent. X1Y1, X2Y2 can be recognized by any of various rearrangements, especially inversions. Sex chromosomes can also be recognized by heterochromatinization of certain regions, supernumerary bands, band dimorphisms and differential expression of the nucleolar organization. In the species where the sex is heterogametic, the sex differential segments exist in the heterozygous condition. This condition restricts recombination between sex chromosomes. Multiple sex chromosome rearrangements as well as undifferentiated sex chromosomes may be found in a single species (Procunier 1982a, McCreadie et al., 1995). The single species with multiple sex chromosomes could be a composite of two or more species each with a unique sex chromosome sequence. The sex linkage may be incomplete which may be due to crossing over (Rothfels 1978, 1980). The condition where the partial sex linkage is known is termed pseudo-partial sex linkage, which involves the coexistence of two structurally identical, inversion bearing chromosomes. In this case only one carries the sex locus (Rothfels 1980, Brookhouse and Adler 2002). Sex chromosomes bring about a difference in most of the species. Sex linked rearrangements on different chromosomes in

non-homologous sex chromosomes are typically associated with different species. Sex lined rearrangements in each of the three member species of *Simulium pictipes* species group are on a different chromosome (Bedo 1975a), may be due to the mobile nature of sex locus (Rothfels 1980, Procunier 1989). It is not necessary to have sex-linked rearrangements of different species on different chromosomes. Different sex related rearrangements on the same chromosomal arm, often within a group of closely related species are associated with separate species as in *Simulium tuberosum* species complex (Landau 1962 and the *Simulium arcticum* species complex. On the contrary, different sex chromosomes do not necessarily mean different species. Many species have sex chromosome polymorphisms where some individuals carry a particular sex-linked rearrangement where other individual carry a different one, although typically on the same chromosomal arm. In *Simulium conundrum* n. sp. in an area less than 3500 km² on Newfoundland's Avalon peninsula, as many as five sex chromosome polymorphisms have been found, although the possibility that some of these polymorphisms corresponds to different species cannot be excluded (McCreadie et al., 1995). *Prosimulium mixtum* and *Prosimulium transbrachium* share the same differentiated sex chromosomes (Rothfels and Freeman 1983).

4.4 Nomenclature

Nomenclature of black flies is specialized in relation to study of cytotaxonomy of mosquitoes, drosophilids and other Diptera. The

present form of nomenclature was first proposed by Rothfels and Dunbar (1953) and further refined and expanded by Basrur (1959), Bedo (1977), Rothfels et al., (1978) and Rothfels (1988). It becomes mandatory to familiarize with this nomenclature for proper understanding of cytotaxonomic literature on black flies.

Most of the black flies have three chromosomes which are numbered as I, II, and III from longest to shortest. These three chromosomes represent approximately 42%, 30%, and 28% respectively of the total complement (Dunbar 1966). Each chromosome is divided into two arms by a presence of a sub-median centromere (C). The centromere often is an expanded region and is deeply heterochromatic. The centromere can be indistinguishable from the remainder of the chromosome bands as in *Simulium pugetense* where the expanded region is absent.

The identification of the centromere has to be done by the presence of ectopic pairing of the centromeric bands and by comparison with the banding sequence of the species whose centromere is known. The expanded centromere region may be transformed as in *Prosimulium*. Such centromeric region is referred to as transformed centromere (Ct) (Basrur 1959).

Each chromosome is designated to have a short (S) and long (L) arms. The difference of short and long arms are readily apparent in chromosome number II and III but the difference in long and short

of chromosome number I is negligible. The short and long arm configuration is consistent across the species, except in *Simulium vittatum* complex where short and long arm of chromosome I are reversed (Rothfels and Featherston 1981) and the species belonging to subgenus *Eusimulium* also exhibit the same. Some species have experienced whole arm interchange, however the arm designations are retained except in subgenus *Helodon*, permitting reorganization that inter changes exist (e.g., IS+IIIL and IL + IIIS in *Simulium decimatum*).

The chromosomal arms have certain land marks that help to readily recognize a particular arm of a particular chromosome. The land marks are characteristics of the chromosomal arms (Rothfels et al., 1978, Rothfels 1988). The land marks are important tool to identify any scrambled section due to one or more inversions. Different chromosomal landmarks are used to designate certain portions of the three different arms. Of the landmarks, the “ring of Balbiani” (BR) (a large puff of distinctive texture) in IIS, the “parabalbiani” (PB) and “3 sharp” in IIL and the “blister” (Bl) in IIIS are amongst the prominent and important ones. There are other numerous markers in IS, IL, and IIIL that are more taxonomically restricted, such as, the “basal 3” and “end marker” (i.e. terminal fine bands in IS). IL is marked with the “Z marker” and “neck”. IIS has the “trapezoid” (T) and nearly universal “bulges (double bubble)” (B or DB) separated by a “shoe string”. IIL is loaded with the “DNA puff” (P), “gray band” (gb), “jagged” (jg), “puffing band”, “saw tooth”, and

"symmetrical". IIIL has the "cup and saucer" and "basal marker". IIIS is endowed with "capsule". The universal landmark found in all black flies is the "nucleolar organizer" (NO). It is a site of heavy ribosomal RNA synthesis and forms a single large nucleolus that appears as an exploded area somewhere in the stained complement. The location of the nucleolar organizer (NO) is species or group specific and, therefore, can provide a simple means of identification. The position of the NO although nearly always constant within a species, it may be highly mobile throughout the family. Therefore, not restricted to any one arm or location. In addition to primary nucleolar organizer, a secondary nucleolar organizer in varying frequencies in some species, e.g., *Simulium anatinum* have been recorded (Rothfels and Golini 1983).

Usually the inversions are designated by a number and the arm in which they are found, for example, IIS-1 as the first inversion, arbitrarily named though often in order of discovery in the short arm of chromosome II. Some authors have also used letter designations (e.g. IIIS-B). Letter P is assigned to designate pericentric inversions. Fixed inversions are represented as underlined (e.g., <u>IIIS-1</u>) or italicized (e.g., *IIIS-1*), distinguishing them from floating inversions (IIIS-1), which are sometimes designated by an abbreviated species name (e.g., IS-1wi in *Simulium williei*). Commas are used for overlapping inversions in order of occurrence (e.g., IS-2, 5), whereas one inversion is included within another or when two inversion are in tandem, a period is used (e.g., IIIL-1.2). Brackets are used for X-

linked inversions or by dashed or by dashed brackets for Y-linked inversions. “ss” (homozygous inverted sequence) are used for three possible configurations of a banding sequence.

A chromosomal map may be constructed by a free hand drawing of the chromosomal complements or a photograph. The standard maps may be constructed representing most central or common sequence of each arm in taxon. Therefore the standard map can be tentative pending additional taxon sampling.

The entire chromosomal complement is divided into 100 approximately equal sections beginning from the telomere of IS to IIIL. The numbers of sections are assigned according to percentage total complement length (%TCL). Sections can be sub-divided in such a way that the bands are designated individually, e.g., 34B2 represent second band in the sub-section B of section 34. Idiograms are often prepared to complement the chromosome maps showing major landmarks and rearrangements.

Although polytene chromosomes exhibit a high degree of taxonomic resolution, various terms has arisen to describe cytologically differentiated taxa such as “cytotypes”, the population that are not reproductively isolated but are cytologically distinct. “Cytoform” are used to the ones as a cytological entity irrespective of whether reproductively isolated or not and does not carry a formal name (Crosskey and Howard 1997). “Cytospecies” is often used to the

population to the ones that are morphologically similar but reproductively isolated. Cases of sibling species of “cryptic species” are special cytospecies.

4.5 Cytotaxonomy

The most important application of Cytotaxonomy has been the illustration of sibling species through fixed inversion differences; this is additionally supported by unique sex chromosome and autosomal polymorphism spectra (Rothfels 1956). Bedo 1979, Tangkawanit et al., 2009, advocated that cytogenetic studies of black flies often reveal the morphologically described species are composed of several cytologically distinct sibling species. These sibling species can be recognized by fixed chromosome inversion differences, differences in sex linked inversions and differences in floating inversions (Rothfels 1979). Different molecular approaches have been applied to differentiate cytospecies but are in vain (Feraday and Leonhardt 1989, Scarpassa and Hamada 2003, Charalambous et al., 2005, Morales-Hojas and Krueger 2009). Study of the sibling species help to gain the idea of reproductive isolation which are inferred by an absence of inversion heterozygotes when two opposite banding sequence are present sympatrically in a considerable number of individuals. Presence of heterozygotes in a population of two valid species provide evidence of hybridization (Rothfels and Nambiar 1981, Boakye et al., 2000). The genetic differentiation at molecular level is a general character of the family Simuliidae (Adler et al., 2010). The low genetic

differentiation of the cytospecies possibly include the ability of long distance migration (Crosskey 1990), Genetic Introgression (Adler et al., 2010) and inadequate variation of the genetic marker used due to recent divergence of the taxa (Scarpassa and Hamada 2003, Conflitti et al., 2010).

Cytotaxonomy, as much it is important to understand the status of the species has its limitations. Whenever possible cytotaxomy should be used in conjunction with other approaches including morphotaxonomy, molecular taxonomy and ecology. No single approach can tackle to solve the issues at species level as it is a continuous natural process especially in case of allopatric species. Reproductively isolated species found at the same site is best demonstrated on the basis of fixed rearrangements. Other limitation of cytotaxonomy to describe a population that differ only in their Y-chromosomes or with differing frequency of autosomal polymorphism or do not differ at all in their chromosomes but yet might be a different species. Gene mutations can cause speciation without apparent chromosomal changes. It is expected that many new species are yet to be discovered on the basis of sex-chromosome polymorphism.

Polytene chromosomes have significantly contributed to the phylogeny of black flies. On the basis of chromosomal rearrangements, especially inversions relative to the standard is taken as evidence of common ancestry (Rothfels 1979). The frequencies of similarities in the polytene chromosomes between the taxa represent their relativeness. With the presence of intermediate species the distant taxa can be correlated. Site specific profiles of autosomal polymorphisms, through polytene chromosomes can be understood, first noted for *Simulium tuberosum* (Landau 1962), that females return to natal ways to breed (Rothfels 1981b) and that movement by ovipositing females occur more readily along the stream then between neighbouring streams (Bedo 1975a). However, all the species do not follow this norm as evidenced in case of *Simuliun venustum*/*verecundum* super complex females do not tend to oviposit predominantly at natal sites (Hunter and Jain 2000).

Chromosomal study also indicates the impacts of environmental insults and the resultant changes in the population over time. It has been observed that heavy metals influence chromosomal features (Sanderson et al., 1982). Polytene chromosome studies have its utility in understanding the quality of the water. In an African species, the presence of a particular inversion has been used to monitor the spread of insecticide resistance (Meredith et al., 1986).

4.6 Utility

Cytotaxonomy is the most important tool for the elucidation of the sibling species on the basis of fixed inversions. The idea of sibling species is further supported by unique sex chromosomes and autosomal polymorphism spectra (Rothfels 1956). Morphologically described species may be composed of several cytologically distinct sibling species. (Bedo 1979, Tangkawanit et al., 2009). Cytogenetic study can help recognize these siblings by fixed chromosome inversion differences; difference in sex-linked inversions and floating inversion differences (Rothfels 1979). Although many molecular approaches have been used to differentiate cytospecies but none of them proven to be successful (Ferady and Leonhardt 1989, Scarpassa and Hamada 2003, Charalambos et al., 2005, Morales-Hojas and Krueger, 2009). The central idea of differentiation of sibling species is on the basis of reproductive isolation which is understood by inversion heterozygotes in a significant number of individuals, when two opposite banding sequence are present sympatrically. Therefore the presence of heterozygotes in a population of two valid species provides the evidence of hybridization (Rothfels and Nambiar 1981, Boakye et al., 2000 a, b). In the family Simuliidae it is suggested that low genetic differentiation at the molecular level is a general character (Adler et al., 2010). There is no certainty to explain the low genetic differentiation of the cytospecies but it is thought to be due to the ability of the long distance migration of these flies (Crosskey 1990), genetic introgression (Adler et al. 2010) and inadequate variation of

the genetic marker that are considered. The genetic structure and diversity pattern of the species are influenced by ongoing gene flow, population fragmentation and population expansion. It is evident that no one methodology is competent enough to understand the population structure in Simuliids. In order to understand the population in the best possible way is to use different approaches such as, morphotaxonomy, cytotaxonomy, molecular taxonomy and ecology in conjunction with each other. Existence of allopatric population poses primary limitations to all the approaches even in a condition where fixed rearrangements are prominent. However, fixed rearrangements differences found at the same site can elucidate reproductive isolation. It is also best to avoid formally naming allopatric populations that bear slight differences. Role of cytotaxonomy is very limited in those populations where there is difference only in Y-chromosome and in the populations that differ in autosomal polymorphism frequency or do not at all differ chromosomally but may be a different species. In these cases demonstration of reproductive isolation cannot be done by a lack of heterozygotes. To argue for species status linking equilibria and heterozygote deficiencies of shared polymorphisms (typically inversions) are taken in consideration (Bedo 1979, Henderson 1986b). Hardy-Weinberg equilibrium is shown to be deviated in case of heterozygote deficiencies, which sometimes is called "Wahlund effect." Speciation and evolution can occur without apparent chromosomal changes, possibly by gene mutations in case of homosequential sibling species. These conditions pose a major

challenge to the taxonomists to discover and recognize a species. While considering above facts many more new species are yet to be discovered in the realm of sex chromosome polymorphisms and homosequential species. Information gathered from polytene chromosomes has enormously contributed to the construction of phylogeny of black flies.

FIGURE: 1a Collection site

FIGURE: 1b Collection site

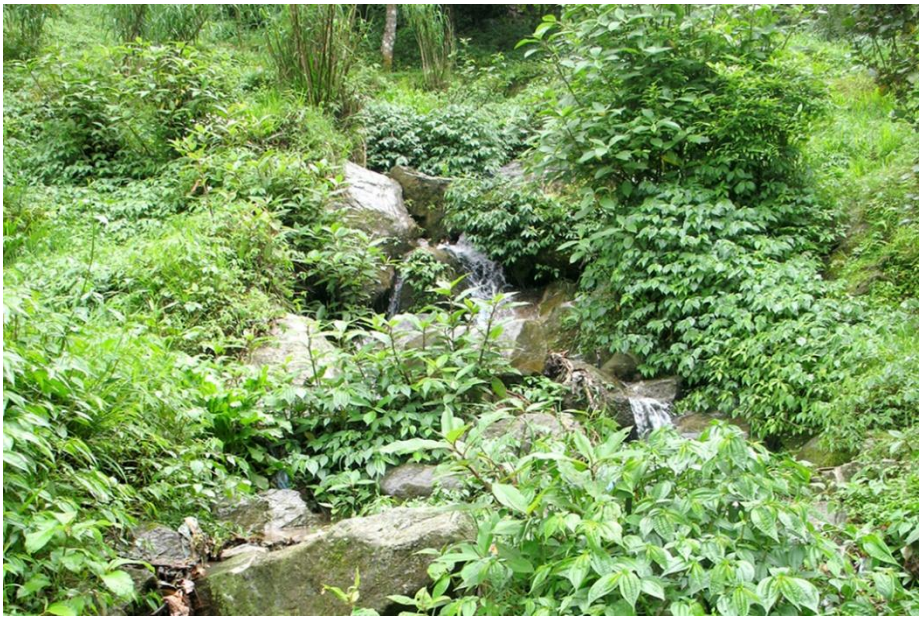

FIGURE: 1c Collection site

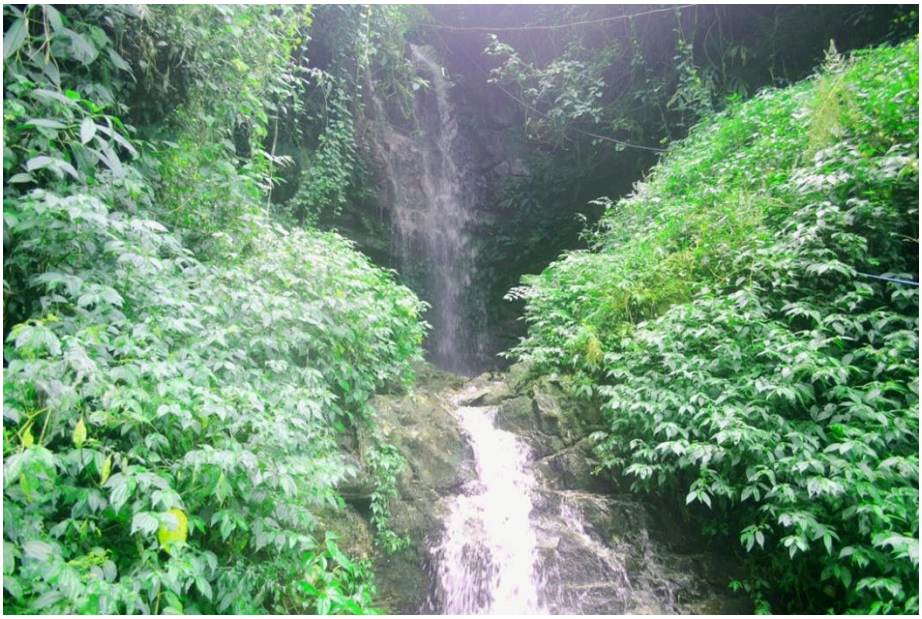

FIGURE: 1d Collection site